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REGULATION OF IMMUNE RESPONSES

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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EDMONTON, ALBERTA

FALL, 1975

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Supervisor

External examiner

Date 18th August, 1975

To my wife, Soo Jeet

ABSTRACT

The expression of humoral immunity involves the production of antigen-specific antibody molecules. This process requires the proliferation of bone marrow-derived (B) and thymus-derived (T) lymphocytes specific for the antigen, as well as the participation of an accessory (A) cell, which is not antigen-specific. Antibody formation and secretion are functions of B cells. Cyclic AMP has been shown to regulate mammalian cell proliferation. It also causes the differentiation and expression of many cell types and thus may be expected to have different effects on the different cell types of the immune system. It was therefore used as a tool to study the regulation of proliferation of antibody-forming cells (AFC), as well as the cellular interactions involved in humoral responses.

AFC to a T cell-dependent (TD) antigen, sheep erythrocytes (SRBC), and a T cell-independent (TI) antigen, polymerized flagellin (POL) from Salmonella adelaide were generated in vitro by culturing CBA/J spleen cells in the Mishell-Dutton or the Diener-Armstrong culture system. High intracellular cAMP levels during the first 12 hr of a 108 hr culture period increased the number of AFC to SRBC and POL. This action of cAMP was antigen-dependent and antigen-specific and could be reversed by cGMP, imidazole, or Con A. Once proliferation of antigen-stimulated lymphocytes was initiated (i.e. 24 hr or later), high intracellular cAMP levels inhibited their proliferation.

The early effects of cAMP on the various cell types involved in the formation of AFC to the TD antigen, SRBC, were determined.

Cyclic AMP inhibited the function of A cells and it did not stimulate the activities of B cells or T "helper" cells. The action of cAMP is exerted through the inhibition of a radiation-sensitive T cell which can regulate the extent of AFC formation. Furthermore, the inhibitory effect of this T regulator cell on AFC production can be overcome by the addition of excess T helper cells. The AFC response to the TI antigen, POL, is also regulated by T cells although AFC formation to this antigen does not require the participation of T helper cells.

The increased number of AFC to SRBC in cAMP treated cultures is not due to a bigger number of antigen-reactive B cells. Instead, the increase can be attributed to a shorter doubling time for AFC as well as a longer period of AFC formation. Since it seems unlikely that the AFC in cAMP treated cultures have a shorter cell cycle time, it is postulated that the increase in number of AFC in treated cultures is due to a greater degree of "conversion" of nonantibody-secreting to antibody-secreting cells. This results from inhibition of T regulator cells by cAMP.

PREFACE

This thesis comprises a major and a minor part. The major part, entitled "Regulation of Immune Responses", was done under the supervision of Dr. Verner H. Paetkau since September, 1972. It is described in full in this thesis.

The minor part of the thesis, entitled "The Nucleotide Sequences and Coding Properties of the Major and Minor Lysine Transfer Ribonucleic Acids from the Haploid Yeast Saccharomyces cerevisiae αS288C", was carried out in the laboratory of Dr. Christopher J. Smith. I first joined this Department in August, 1971 as a graduate student of Dr. Smith. At that time, the work by Dr. Smith on the nucleotide sequence of the major lysine tRNA of αS288C was nearing completion. Dr. Arthur N. Ley assisted Dr. Smith in the nucleotide sequence studies and Mr. Perry D'Obrenan was responsible for preparing the ^{32}P-labelled lysine tRNA. My contribution to that project was the determination of the nucleotide sequences of partial digests of the major lysine tRNA with ribonuclease T1 and ribonuclease A. In early February, 1972, Dr. Smith, Mr. D'Obrenan and I discovered that αS288C has a minor lysine tRNA. Later that month Dr. Smith was killed in a tragic mountaineering accident. But Mr. D'Obrenan and I decided to continue with the project and we further characterized the minor lysine tRNA. This tRNA turned out to be identical to a lysine tRNA from Bakers' yeast, the sequence of which was determined by Dr. J. T. Madison and coworkers. Before starting work in Dr. Paetkau's laboratory, I was responsible for publishing the work on the lysine tRNAs in the Journal of Biological Chemistry, a reprint of which is included as an appendix to the main thesis.

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I am grateful to my supervisor, Dr. Verner H. Paetkau, for guidance and helpful discussions throughout the course of this work. I am also indebted to Dr. Erwin Diener, Chairman of the Department of Immunology, for kindly allowing me to use his laboratory facilities and materials during the past three years.

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LIST OF ABBREVIATIONS AND GLOSSARY

A cell	accessory cell; this term used synonymously with "macrophage"
AFC	antibody-forming cell(s)
AP	aminophylline, an inhibitor of cyclic AMP phosphodiesterase
Ara-C	cytosine-arabinoside, a reversible inhibitor of DNA synthesis
ATXBM	adult mice that have been thymectomized, lethally irradiated, and reconstituted with syngeneic (identical genetically) bone marrow cells
B cell	bone marrow-derived or "bursa-equivalent" lymphocyte
8-Br-cGMP	8-bromo-guanosine-3',5'-cyclic monophosphate
BSS	balanced salts solution
carbachol	carbamylcholine chloride, a stimulator of guanylate cyclase
carrier	the part of the antigen that is recognized by thymus-derived lymphocyte(s)
CBA/J	inbred mouse strain
CBA/CaJ	inbred mouse strain, related to CBA/J
CMI	cell-mediated immunity, a function of thymus-derived lymphocytes
C_H	constant region of immunoglobulin heavy chain
C_L	constant region of immunoglobulin light chain
complement	serum proteins, when used in collaboration with antibodies specific for a foreign cell, cause the cytolysis of that cell
Con A	concanavalin A, a mitogen for thymus-derived lymphocytes
cAMP	adenosine-3',5'-cyclic monophosphate
cGMP	guanosine-3',5'-cyclic monophosphate

cytotoxic lymphocyte	thymus-derived lymphocyte which has the ability to kill target cells to which it has been sensitized; the killing process does not require the presence of complement
DBA/2J	inbred mouse strain
dbcAMP	N^6-$O^{2'}$-dibutyryl-adenosine-3',5'-cyclic monophosphate
dbcGMP	N^2-$O^{2'}$ dibutyryl-guanosine-3',5'-cyclic monophosphate
FCS	fetal calf serum
hapten	a chemically defined antigenic determinant which is recognized by bone marrow-derived lymphocytes
H chain	heavy chain (class = γ, μ, α, δ, or ε) of immunoglobulin
HI	humoral immunity
H-2	mouse major histocompatibility locus
Ig	immunoglobulin
IgG	7S immunoglobulin (L chain = κ or λ; H chain = γ)
IgM	19S immunoglobulin (L chain = κ or λ; H chain = μ)
IU	immunocompetent units; defined as the minimum number of interacting cell types required to generate an antibody response to an antigen
L chain	light chain (class = κ or λ) of immunoglobulin
Ly	thymus-derived lymphocyte surface marker
LPS	bacterial lipopolysaccharide
MEM	minimum essential medium
2-Me	2-mercaptoethanol
mean $\pm$ SEM	arithmetic mean $\pm$ standard error of the mean
nu/nu	congenitally athymic mice
PBS	phosphate buffered saline

poly A:U	a double-stranded hybrid of polyriboadenylic acid and polyribouridylic acid
PFC	plaque-forming cell, equivalent to antibody-forming cell to sheep erythrocytes
PHA	phytohemagglutinin, a mitogen for thymus-derived lymphocytes
POL	flagellar protein from Salmonella adelaide; a thymus-independent antigen
PVP	polyvinylpyrollidone, a mitogen for bone marrow-derived lymphocytes
PWM	pokeweed mitogen, a mitogen for thymus-derived and bone marrow-derived lymphocytes
SIII	pneumococcal polysaccharide type III
SRBC	sheep erythrocytes, a thymus-dependent antigen
T cells	thymus-derived lymphocytes
TD antigen	T cell-dependent antigen
TI antigen	T cell-independent antigen
(T,G)-A--L	the synthetic multichain polypeptide poly-(tyrosyl-glutamyl)-poly-D,L-alanyl-poly-lysine
θ (or Thy-1)	thymus-derived lymphocyte surface marker
V_H	variable region of immunoglobulin heavy chain
V_L	variable region of immunoglobulin light chain
tolerant	an immunologically unresponsive state

CHAPTER I

INTRODUCTION

A. General

The distinguishing feature of the vertebrate immune system is its ability to differentiate self constituents from determinants on foreign macromolecules, or antigens. The ability to respond to foreign antigens forms the basis on which the vertebrate organism can guard its identity and protect itself against harmful foreign agents, e.g. pathogens. One means by which this is achieved is through the production of antibody molecules capable of specific binding to foreign antigens. Since the immune system recognizes a vast number of foreign antigens it follows that the number of different antibody molecules synthesized is correspondingly very large. The central problem in immunobiology, therefore, is how the information for synthesizing a large number of antibody molecules is generated, stored, and expressed in a controlled fashion. Recent studies on myeloma proteins have demonstrated that the basis for antibody activity lies in the amino acid sequence of immunoglobulins, and that the genetic information for many of their structures, at least, is transmitted from parents to progeny. Futhermore, certain genes are now known to control immune responses. And finally, cells with different functions in immune responses have been identified. These include cells able to recognize antigens, other cells able to inhibit or stimulate the responses of antigen-sensitive cells, and effector cells which execute the neutralizing mechanisms of immune responses.

An area of central importance is the mechanism by which

lymphocytes are triggered into proliferation or rendered tolerant (i.e. unresponsive) following their interaction with the appropriate antigen. Evidence, to be described below, indicates that antigen-lymphocyte interactions occur at the cell's plasma membrane, through immunoglobulin (Ig) receptors which link the genetic determination of the cell to the stimulating antigen. Little is known as to how cell surface interactions are translated into intracellular signals that would eventually determine the pathway of differentiation of the stimulated lymphocyte. Direct studies on this have been difficult because the cells reactive to a given antigen form a very small proportion of the total lymphocyte pool. Therefore work on this question has concentrated on agents or mechanisms which are generally able to alter proliferation and differentiation of mammalian cells. An attractive candidate for this role is adenosine-3',5'-cyclic monophosphate (cAMP), because in addition to regulating cellular metabolic processes it has been shown to play important roles in the control of mammalian cell proliferation and differentiation.

B. The Genesis of Lymphocytes

A schematic overview of the developmental pathway of the lymphoid system is given in Fig. 1.1. The lymphocyte originates from hemopoietic stem cells (Wu et al., 1967) which are first found in the blood islands of the embryonic yolk sac (Tyan and Herzenberg, 1968; Moore and Metcalf, 1970). The stem cells appear not to be specificity restricted and lack the characteristic surface antigens found in mature lymphocytes. From the embryonic yolk sac they migrate, first to the liver and later to the bone marrow and spleen (Moore and

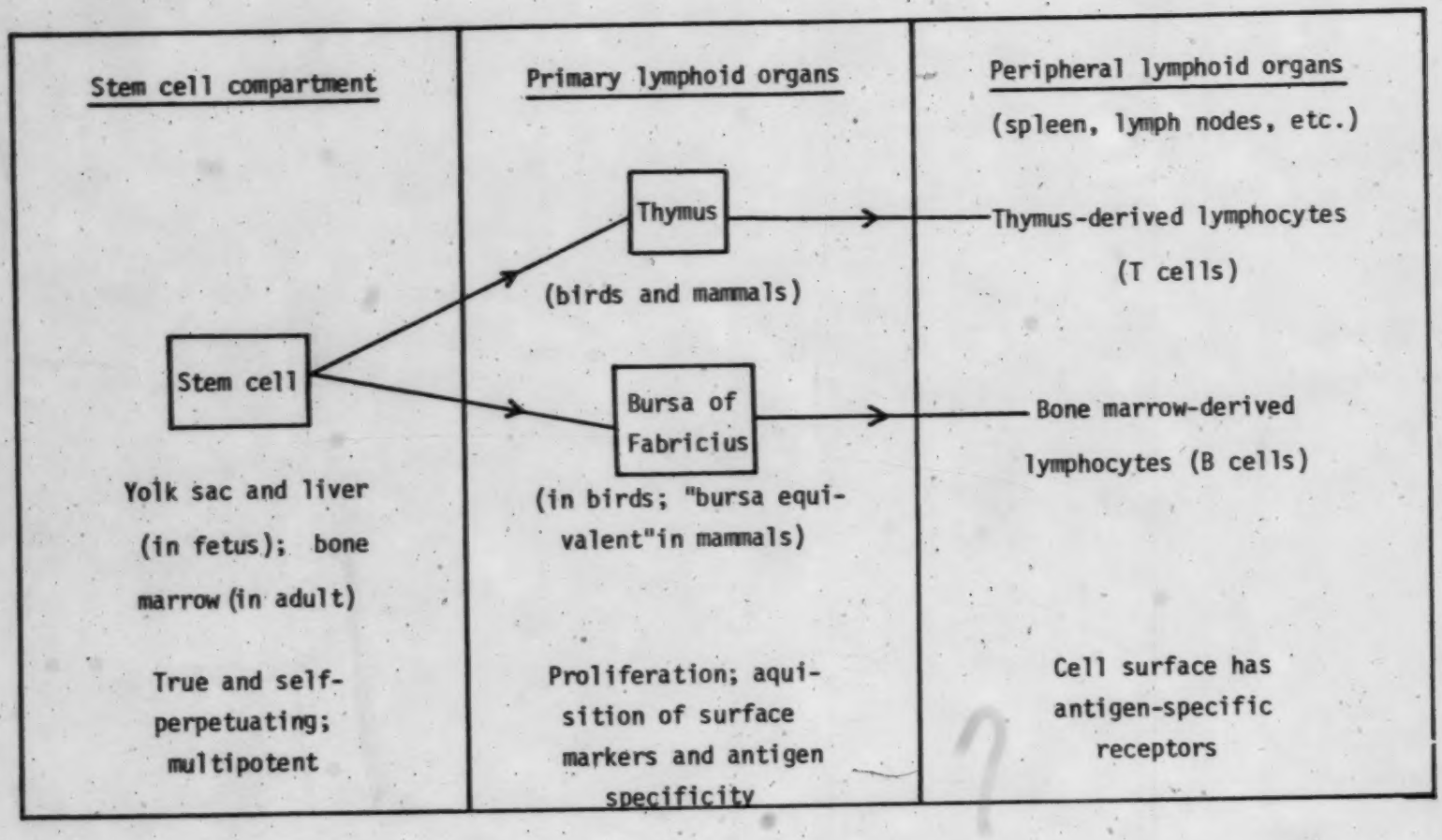


Fig. 1.1. A scheme for the development of immunocompetent lymphocytes. The maturation of stem cells into immunocompetent lymphocytes is independent of antigen. Further differentiation of the mature lymphocytes into effector cells in immunity requires antigenic stimulation.

Owen, 1965; Moore and Owen, 1967). The hemopoietic stem cells persist in the bone marrow and to a lesser extent in spleen throughout adult life and retain their embryonic potential (Moore and Owen, 1967). The maturation of stem cells into immunocompetent lymphocytes, i.e. lymphopoiesis, takes place in the "primary lymphoid organs". In birds these organs have been identified as the bursa of Fabricius and the thymus (Cooper et al., 1966). In mammals lymphopoiesis takes place in the thymus and a diffused "bursa-like" environment.

The thymus is the organ in which immature lymphocytes differentiate, independent of antigenic influence, to become the so-called thymus-derived lymphocytes, or T cells. During differentiation thymic lymphocytes undergo sequential changes in expression and organization of surface antigens. In the mouse, the development in the thymus of surface antigens such as θ (or Thy-1), TL, and Ly have been followed (Aoki et al., 1969; Komuro and Boyse, 1973). T cells are then seeded to secondary or peripheral lymphoid organs such as the spleen and lymph nodes (Nossal and Ada, 1971). Maturation of stem cells into B lymphocytes proceeds in the bursa of Fabricius in birds (Cooper et al., 1966). In mammals the site of stem cell maturation into B cells is still unknown (Nossal and Ada, 1971; Miller and Phillips, 1975). Mature B cells are found in the germinal centers of spleen and the paracortical follicles of lymph nodes.

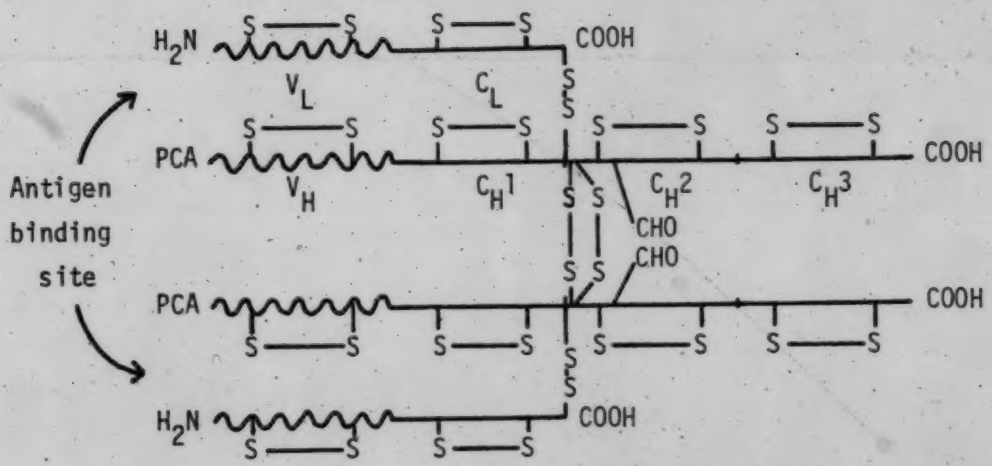
The differentiated B and T cells proliferate only slowly in the absence of a further stimulus. Their centrally important characteristic is that they are specificity-restricted, i.e. they bear receptors specific for a single antigen.

C. Receptors and Immunoglobulins

The specificity of the receptor on a given cell corresponds to the specificity of the antibody molecules the cell can secrete when properly stimulated. Thus, the T or B cell has become unipotent during its maturation and is now determined with respect to its antigen-recognizing capacity. The general area of this thesis is the further differentiation of mature B cells into proliferating antibody-secreting effector cells in the primary immune response.

B cells bear immunoglobulin (Ig) receptors which are antibody molecules. The basic unit for all antibody molecules is essentially similar and is diagramatically represented in Fig. 1.2. This unit comprises two light (L) and two heavy (H) polypeptide chains. The L chain has a variable (V_L) and a constant (C_L) region each about 110 amino acid residues in length. The H chain is twice the length of the L chain and consists of one V and three C regions (V_H, C_H1, C_H2, C_H3, respectively). One V_H and one V_L together constitute one antigen binding site. In the human sera, the L chain could be either of the κ or λ class, whereas the H chain could be either of the γ, μ, α, δ, or ξ class, the resulting Ig being referred to as IgG, IgM, IgA, IgD, and IgE, respectively. These classes differ from one another in properties such as size, charge, number of antigen binding sites, position and number of disulphide bonds, length and composition of the individual polypeptide chain and carbohydrate content.

The nature of T cell receptors is not as well characterized as are B cell receptors. Marchalonis (1974) has provided evidence that T cells possess 7S IgM-like receptors which show specificities for antigens. However, an apparent paradox exists, because although the



PCA = pyrollidonecarboxylic acid

CHO = carbohydrate

Homology units	
V-region	C-region
V_L	C_L
V_H	C_H1
	C_H2
	C_H3

Fig. 1.2. A schematic representation of the structure of the 7S IgG molecule.

presence of Ig receptors on T cell membranes can be functionally demonstrated by inhibition of antigen binding or "hot antigen suicide", direct studies with labelled anti-Ig reagents yield little or no binding (for references, see Marchalonis, 1974). More studies are therefore required to confirm the identity of T cell receptors.

D. Theories of Antibody Diversity

The immune system is capable of making a very large number of antibody molecules with different antigen binding specificities. The question then arises as to how the genetic information required for such a large number of polypeptide chains is stored and generated. Two theories have been put forward. The germ line theory suggests that each antibody molecule must be coded for by a germ line gene (Szilard, 1960; Dreyer and Bennett, 1965; Hood and Talmage, 1970). On the other hand, the somatic theory suggests that the information of antibody molecules may be generated from a limited number of germ line genes by mutational or recombinational processes during development (Lederberg, 1959; Edelman and Gally, 1967; Smithies, 1967)

One approach to understanding mechanisms of information storage and expression is the comparative analysis of individual gene products. Detailed chemical studies of normal antibody molecules are difficult to do because serum proteins are heterogeneous. Fortunately, homogeneous Ig, produced by myeloma cells, can be isolated. Myeloma globulins are Ig that appear in the plasma of patients or animals with multiple myeloma, a tumour of antibody-forming cells (AFC). The homogeneous Ig produced by myeloma cells can comprise up to 95% of serum Ig. These proteins therefore permit a detailed

comparative study of antibody structures. On the basis of available amino acid sequence data, Hood and Prahl (1971) have been able to construct genealogical patterns for the V regions of κ light chains of mouse and human myeloma proteins. They concluded that the V regions of κ light chains probably arise from one primordial gene and the diversity of the V region is generated by gene duplication and selection during evolution. Thus each modern individual inherits the genetic information for the myeloma proteins, at least, through germ line genes. The information for the immune system thus appears to be encoded as germ line genes.

On the other hand, it is not obvious that all antibody specificities are transmitted through the germ line. Quite strong arguments for the occurrence of somatic mutation have been made by Cohn and Tonegawa (Cohn et al., 1974; Tonegawa et al., 1974), but these go beyond the scope of this dissertation. The controversy is clearly unresolved at present. What both theories have in common, of course, is that the antigen specificity of a mature, determined, B or T cell is based on expressed information in the chromosome.

E. The Clonal Selection Theory

In the course of an immune response, specific antibody molecules are produced only to the stimulating antigen. Therefore, there must exist a selection mechanism whereby an antibody-forming cell precursor is induced to proliferate and secrete antigen-specific antibody molecules following its encounter with the antigen. This problem of recognition and amplification was first considered in detail by Ehrlich (1900). He envisioned lymphocytes as pluripotential,

possessing diverse cell surface receptors. When antigen was introduced into the organism, it interacted with complementary receptors and caused them to be released from the cell. This release triggered the synthesis of more of that particular receptor in the corresponding cell. In the 1920s and 1930s it became apparent that the immune system is capable of an extremely broad range of response (Landsteiner, 1947). It appeared unreasonable that the vertebrate organism could have such a large number of preformed antibody molecules as required by the selective theory. This view led to the instructionistic theory (Breinl and Haurowitz, 1930), which proposed that the antigen must provide the immune system with the information required for specific antibody synthesis. Pauling (1940) reduced the instructionistic interpretations to molecular terms by postulating that the antigen served as a template which permitted the antibody polypeptide chain to fold about it in a complementary fashion. We now know that this theory is wrong because the three dimensional structure of a polypeptide is determined by its primary amino acid sequence; denaturation and renaturation of an antibody molecule in the absence or presence of antigen yields the same antibody molecule (Epstein et al., 1963). Another objection to the instructionistic theory, raised at that time, was that it could not satisfactorily account for immunological memory, i.e. the ability of the immune system to react more vigorously to a second encounter with the same antigen.

The first modern selectionistic theory was proposed by Jerne (1955). He postulated that antibodies of all potential specificities were contained in the serum of each individual. An antigen, upon interaction with its complementary molecule, formed an antigen-

antibody complex which was recognized by the lymphocyte. This complex then stimulated the synthesis of specific antibody molecules in the lymphocyte. This selectionistic approach was further expanded by Burnet (1959) who suggested that the "unit of selection" was the lymphocyte itself rather than the humoral antibody molecule. In this view, each lymphocyte carried only one type of cell surface receptor. The antigen would then interact only with lymphocytes possessing complementary cell surface receptors. This interaction would result in the proliferation of that clone into AFC, secreting antibody with the same antigen binding specificity. Direct confirmation of this theory came from studies of antigen binding cells. Naor and Sulitzeanu (1967) were the first to show that only a very small proportion of lymphocytes was able to bind specifically to an antigen labelled with ^{125}I. More experimental support for the clonal selection theory came from the studies of Ada and Byrt (1969). They showed that when normal spleen cells were incubated with Salmonella flagellar protein type 1338, labelled to a very high specific activity with ^{125}I, the antigen-reactive clone for type 1338 was specifically abrogated through localized ^{125}I decay ("antigen suicide"); the same cell population retained its ability to respond to another Salmonella antigen (type 870). Similarly, Humphrey and Keller (1970) were able to show that (T,G)-A- -L (the synthetic multichain polypeptide poly-(tyrosyl, glutamyl)-poly-D,L-alanyl-poly-lysyl) labelled with radioactive ^{125}I to a high specific activity, was non-immunogenic, whereas (T,G)-A- -L labelled with nonradioactive ^{127}I was immunogenic.

F. T and B Cells in Immune Responses

The introduction of foreign antigens into an animal usually results in the production of antibodies and/or reactive cells, both of which can circulate in the blood and react specifically with antigen. In general, immune responses which can be transferred to another animal by means of serum from a sensitized donor (containing antibody) are termed humoral immunity (HI) whereas those than can be transferred by sensitized cells, but not by serum, are called cell-mediated immunity (CMI). A summary of the characteristics and functions of humoral and cell-mediated immunity is given in Table 1.1.

The evidence that T cells are required for cell-mediated and B cells for humoral immunity came from studies in birds (Cooper et al., 1966; Warner and Szenberg, 1964) and rodents (Miller, 1961). It was found that removing the thymus from an embryo or newborn impaired the CMI of the animals when they grew up, but had much less effect on HI. On the other hand, removal of the bursa of Fabricius at hatching impaired the bird's ability to make antibody but had little effect on CMI. Patients with immunological deficiency diseases also show this dichotomy. Thus, patients deficient in B cells (e.g. Bruton-type congenital agammaglobulinemia) do not make antibody but have normal CMI, whereas patients with congenital thymic aplasia (e.g. Di George's syndrome) show markedly impaired CMI but can make relatively normal amounts of antibody in response to some antigens (reviewed by Good et al., 1971). More recently, it was shown that congenitally athymic (nu/nu) mice were capable of accepting for their lifetime skin grafts from distantly related mammals and birds as well as skin grafts from reptiles and amphibians; thymus implantation

Table 1.1

Characteristics and functions of humoral and cell-mediated immunity

A) Humoral immunity:

1. Is a function of B cells,
2. Is effected by specific antibody molecules,
3. Inactivates foreign materials, e.g. bacterial toxin,
4. Produces immediate hypersensitivity (e.g. hayfever, drug allergy),
5. Regulates the function of both B and T cells by competing with lymphocyte receptors for antigenic determinants,
6. May aid in the localization of antigen on appropriate lymphoid tissues by antibody,
7. Produces antibody-antigen complexes which induce immune paralysis (tolerance).

B) Cell-mediated immunity:

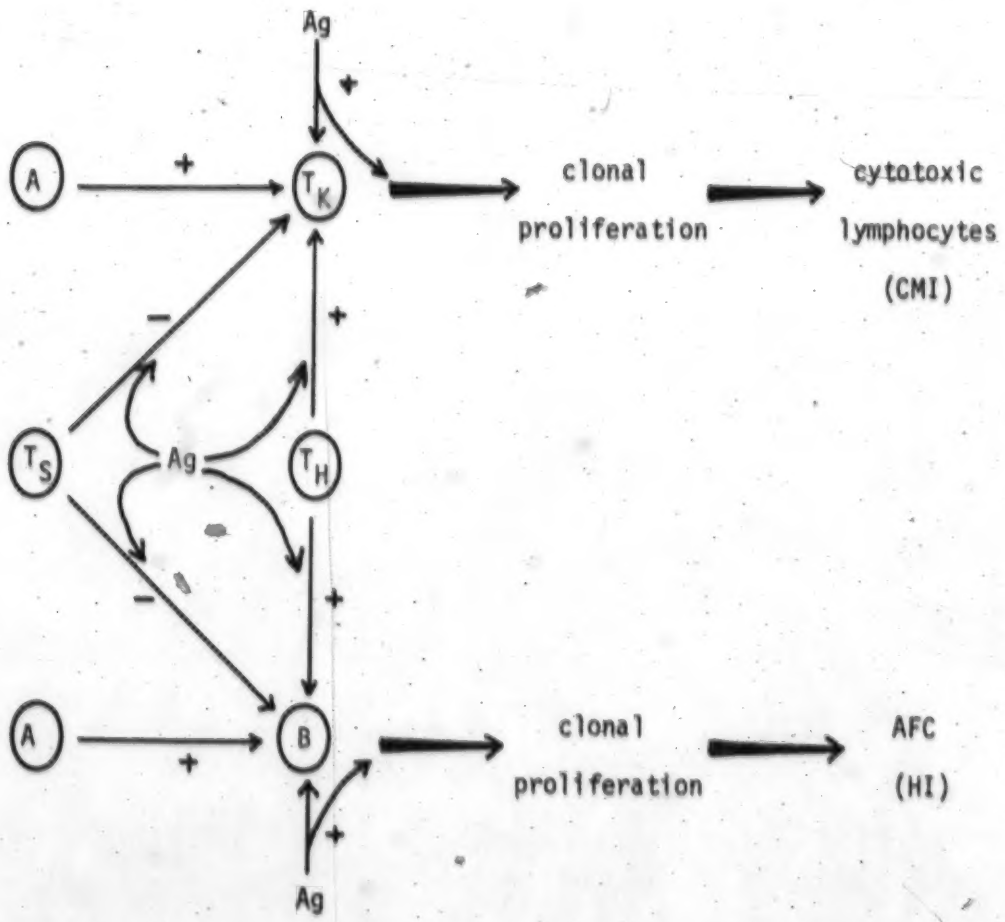
1. Is a function of T cells,
2. Is effected by cytotoxic lymphocytes,
3. Includes production of antigen-nonspecific factors or lymphokines (e.g. migration inhibition factors, chemotactic factors, cytotoxic factors, and mitogenic factors),
4. Produces delayed hypersensitivity,
5. Is the basis for rejection of foreign tissues,
6. Is the mechanism for graft-versus-host responses (where transplanted foreign lymphoid cells respond against the antigens of the recipient, often resulting in recipient death),
7. Produces immunity to various microbes.

into athymic mice enabled them to reject such foreign skin grafts (Manning et al., 1973).

In vitro studies confirmed that cytotoxic lymphocytes are of thymic origin. During maturation in the thymus T cell precursors acquire the θ antigen (Reif and Allen, 1964). Antibodies specific for the θ antigen can be used, with complement, to destroy T cells selectively (Raff, 1969). Using this technique Cerottini et al. (1970) demonstrated that cytotoxic lymphocytes and their precursors carry the θ marker. This is in contrast to antibody-secreting cells. It is therefore clear that target cell destruction by killer cells is a specific function of T cells whereas cells that form antibody are of non-thymic origin. Evidence that B cells are precursors of AFC was conclusively provided by Nossal et al. (1968). By reconstituting lethally irradiated mice with mixtures of chromosomally marked bone marrow cells and normal thymus cells, they were able to demonstrate that all the AFC were of bone marrow origin.

G. Cellular Interactions in Humoral and Cell-Mediated Immunity

The cellular interactions involved in the generation of cell-mediated and humoral immunity are complex and not fully understood (Fig. 1.3). It is clear that three cell types are required for the initiation of an antibody response to sheep erythrocytes (SRBC) and to several serum protein antigens in mice: a B cell, a T "helper" cell and an accessory (A) cell (reviewed by Miller and Mitchell, 1969; Claman and Chaperon, 1969; Taylor, 1969; and, Talmage et al., 1970). The A cell is a glass-adherent, phagocytic (Mosier, 1967) and radioresistant (Roseman, 1969) cell. The terms



T_K = cytoxic lymphocyte ("killer cell") precursor
T_H = T helper cell
T_S = T regulator ("suppressor") cell
B = AFC precursor

Ag = antigen
A = accessory cell
\+ = stimulation
\- = inhibition

Fig. 1.3. A simplified scheme of the cellular interactions during cell-mediated and humoral immunity.

A cell and macrophage have been used interchangeably in the literature. The induction of a cytotoxic lymphocyte response takes place in an analogous manner. The cell types involved are the cytotoxic lymphocyte precursor (which is a T cell), a helper cell and an accessory cell (Cantor and Asofsky, 1972; Wagner, 1973). More recently, a T cell with suppressor activity for the induction of humoral and cell-mediated immunity has been demonstrated (reviewed by Gershon, 1974). The T suppressor and T helper cells are probably derived from different T cell subpopulations (Dutton, 1973; Scavulli and Dutton, 1975). One major advantage of the participation of multiple cell types is that it allows these responses to be regulated at several levels.

The cellular interaction most extensively studied is the collaboration between T and B cells (reviewed by Katz and Benacerraf, 1972). Claman et al. (1966) were the first to show that thymus and bone marrow cells act synergistically to give an antibody response. The bone marrow-thymus interaction was further substantiated by the work of Miller and associates (Miller and Mitchell, 1968; Nossal et al., 1968). Other evidence for T-B collaboration came from studies of antibody responses against chemically-defined determinants called "haptens". A hapten by itself cannot elicit an antibody response, but must be covalently linked to a "carrier" to be immunogenic. Furthermore, the nature of the carrier portion of the antigen is important in immunogenicity. In general, immunization is much better if the carrier is a strong antigen. Mitchison first pointed out that the "carrier effect" may depend on T-B cell collaboration. He showed that the antibody production against a hapten was aided by "helper cells" with immunological reactivity to the carrier

(Mitchison, 1971a). These carrier-primed cells were subsequently shown to be T cells since they were killed by anti-θ serum in the presence of complement (Raff, 1970).

However, not all antigens are hapten-carrier complexes. A number of antigens exist which can stimulate B cells directly without the cooperation of T cells. These antigens are usually repeating polymers and include the polymerized flagellin (POL) of Salmonella adelaide, levan from Corynbacerium levaniformis, dextran, pneumoccal polysaccharide type III (SIII), bacterial lipopolysaccharide (LPS), and polyvinyl pyrollidone (PVP) (Coutinho and Möller, 1973).

H. Models of B Lymphocyte Activation

Several hypotheses have been advanced to explain the mechanism underlying T-B cell collaboration. Mitchison (1971b) suggested that T cells have carrier-specific IgX receptors (implying an Ig-like structure without defining the antibody class). His antigen-focusing hypothesis suggests that the role of T cells is to bind the carrier portion of the antigen and present the haptenic part of the antigen in proper orientation and concentration to B cells. Experimental support for antigen-focusing, also known as the "matrix hypothesis", came from the observation that the monomer of POL was T cell-dependent (TD) whereas the polymer was T cell-independent (TI) (Feldmann and Basten, 1971). Further, a TD antigen, with one chemically-defined haptenic determinant, when covalently linked to Sepharose beads or polyacrylamide, was converted to a TI antigen (Feldmann et al., 1974). It thus appeared that T cells act by making monomers behave like polymers. The matrix hypothesis was further

elaborated by Feldmann and Nossal (1972) who suggested that antigen-activated T cells secrete monomeric IgM, referred to as IgT. Many IgT-antigen complexes will bind to the surfaces of A cells to form a matrix, where the antigen is properly orientated and present in sufficient concentration to activate B cells. Evidence against this hypothesis came from the observations that monomeric antigen induces responses in the absence of T cells (Hunter and Munro, 1972), that some polymeric antigens are T cell-dependent in rats (Steward, 1971), and that TI antigens do require a small number of A cells (Lee et al., 1975), indicating that a matrix is insufficient for induction.

An alternative hypothesis was advanced by Bretscher and Cohn (1968). Their "two signal" model suggests that the binding of antigen to B cells induces a tolerogenic signal, since haptens coupled to nonimmunogenic carriers are tolerogenic (reviewed by Bretscher, 1972). B cell triggering will occur only when a T cell-derived associative antibody (equivalent to IgX) is also present to provide the inductive signal. Polymeric antigens, because of their structure, were postulated to require very small amounts of associative antibody to trigger B cells. However, this argument is weakened by the recent finding that B cells in nu/nu spleen cultures can proliferate in the absence of T cells when stimulated by a TD antigen (Dutton, 1975).

The starting point for the "one non-specific signal" hypothesis of Coutinho and Möller (1974) was the observation that all the TI antigens they tested were found to be polyclonal B cell activators (mitogens) in terms of ^{3}H-thymidine uptake and antibody responses. They postulated that B cells are activated by non-specific

signals delivered to the cells by surface structures which are not immunoglobulin receptors. The immunoglobulin receptors serve an important passive focusing function and permit selective binding of the antigen to the specific cells. The function of immunoglobulin receptors is to ensure the specificity of the response and not to initiate the response. The actual triggering event is caused by signals delivered either by the antigen itself (but not by its antigenic determinants) in the case of TI antigens, or by factors released from T cells and/or macrophages in the case of TD antigens. However, the mitogenicity of TI antigens is weak and the observed response does not appear to be of sufficient magnitude to provide a strong indication of biological relevance (Greaves et al., 1974).

It is clear from the above discussion that none of the above hypotheses accounts for all the observations relating to B cell triggering. Perhaps the understanding of T-B cell collaboration will become clearer when the structure of antigen-specific T cell factors is better understood. Work along this line has shown that an antigen-specific factor to (T,G)-A--L is coded for by a gene in the mouse major histocompatibility complex (H-2); this factor (M. Wt. = 50,000) is adsorbed by antigen and anti H-2, but not by anti-Ig (Munro and Taussig, 1975).

What is apparent is that B cell stimulation does not require that antigens enter the cell (Feldmann et al., 1974). Further, there are antigen-specific T cell factors which are necessary for complete B cell responses to TD antigens. Similar factors, of non-specific nature, also contribute to B cell stimulation by antigens. And finally, some antigens can elicit B cell responses in the apparent

absence of T cells.

I. Activation of Lymphocytes by Mitogens

Biochemical analysis of specific immune responses has been difficult since the number of cells that are responsive to a given antigen forms a very small fraction of the total lymphocyte population. Recently, a variety of agglutinins of plant origin has been found to induce proliferation in a large proportion of the lymphocyte pool (reviewed by Greaves and Janossy, 1972), thus providing a model for biochemical analysis. These mitogens include phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM). The mitogens PHA and Con A preferentially stimulate T cells whereas PWM can stimulate both T and B cells (Greaves and Janossy, 1972). Mitogens specific for B cells include LPS, levan, SIII, PVP, dextran, and POL (Coutinho and Möller, 1973).

The binding of mitogens to the lymphocyte surface constitutes an essential but insufficient requirement for the initiation of lymphocyte proliferation. Thus, B cells can bind as much Con A as do T cells, but are not stimulated (Stobo et al., 1972). Internalization of the mitogen into the cell is also not necessary for lymphocyte triggering (Greaves and Bauminger, 1972; Andersson and Melchers, 1973). Thus, there must exist a mechanism by which cell surface signals are translated into intracellular signals responsible for inducing mitosis in the lymphocyte. The nature of such a mechanism is still poorly understood.

Extensive reorganization of cell surface receptors for Con A, PHA, and PWM have been shown to occur following the binding

of these mitogens to lymphocytes, i.e. "patching" followed by the "capping" of bound receptors (Greaves and Janossy, 1972). A correlation between mitogen binding and membrane fluidisation has been described. Furthermore, concentrations of mitogen which were too high to stimulate the cells ("supramitogenic" levels) also failed to induce the immediate fluidisation of the cell membrane (Barnett et al., 1974). Capping by itself does not constitute a trigger since B cell receptors for Con A, a T cell mitogen, can also cap in the presence of Con A (Greaves and Janossy, 1972).

In addition to surface events, changes in cellular metabolism have also been studied. The addition of PHA generates several biochemical changes in the cell within the first hour: 1) an increase in the incorporation of $^{32}P_i$ into phosphatidylinositol in the membrane (Fisher and Mueller, 1971); 2) an increase in the cell uptake of RNA, protein, and lipid precursors (Pogo, 1969; Peters and Hausen, 1971); 3) an increase in K^+ (Quastel et al., 1970) and Ca^{++} transport (Maino et al., 1974); 4) changes in intracellular cyclic GMP (Hadden et al., 1972) and cyclic AMP levels (Smith et al., 1971a; Krishnaraj and Talwar, 1973; Webb et al., 1973); 5) acetylation of histones (Pogo et al., 1966) and binding of acridine orange dye to the deoxyribonucleoprotein (Killander and Rigler, 1969); 6) phosphorylation of nuclear basic proteins (Kleinsmith et al., 1966; Cross and Ord, 1971); and 7) an increase in RNA and protein synthesis (Bach and Hirschhorn, 1963; Rubin and Cooper, 1965; Pogo et al., 1966). DNA synthetic activity does not increase until about 20 hr later and is correlated with an increase in DNA polymerase activity (Loeb et al., 1968). The relative importance of these observed metabolic changes

in the initiation of lymphocyte proliferation has not been established. Very little evidence exist as to which factors are causes, and which are results, of stimulation.

J. Cyclic Nucleotides as Intracellular Mediators of Lymphocyte Activation

The large number of biochemical changes observed following the addition of a mitogen to lymphocytes led to the speculation that the mitogen may activate certain key intracellular mediator(s) (Watson, 1975). Cyclic AMP is an attractive candidate in this regard because: 1) it is formed by the activation of a membrane-bound enzyme, 2) it occupies a key position in cellular metabolic processes, 3) it is important in regulating cell proliferation, and 4) it is involved in mammalian cell differentiation.

Cyclic AMP was discovered by Sutherland and Rall (1957) in the course of investigating factors controlling the degradation and synthesis of glycogen in liver cells. The scheme for the formation and breakdown of cAMP as well as its mode of action in liver cells is shown in Fig. 1.4. The net result of the binding of glucagon and epinephrine to liver cell membrane is the conversion of liver glycogen into glucose molecules. It is now clear that the actions of many other hormones are also mediated by cAMP (Robison et al., 1968).

Evidence that cell proliferation is regulated by cAMP came from measurements of cellular cAMP levels in cultured fibroblasts at various stages of their growth cycle. It was found that the highest cellular cAMP levels were attained when the cells were arrested

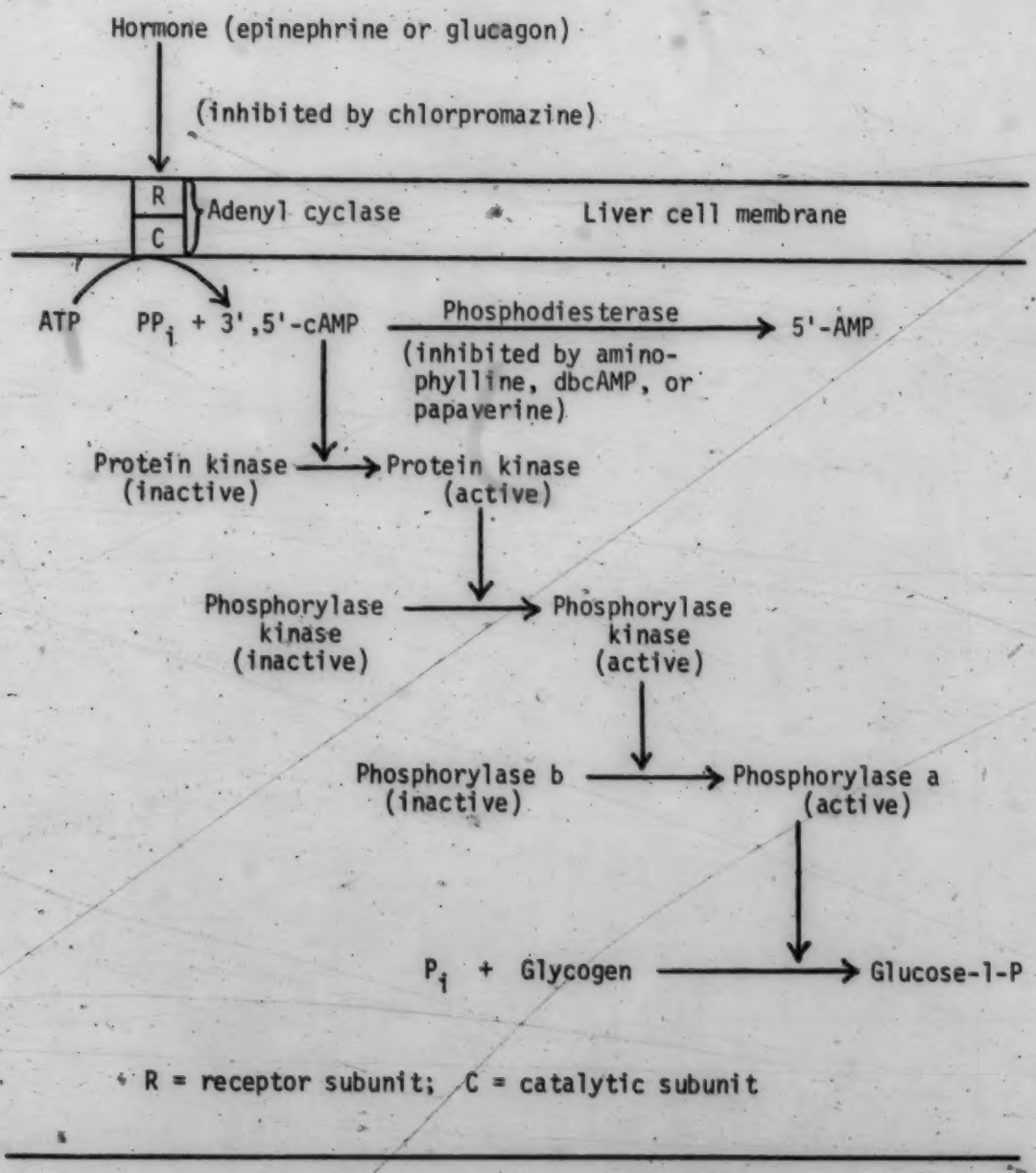


Fig. 1.4. A scheme for the formation and degradation of cAMP and its role in the regulation of glycogen levels in liver cells.

at confluency (Burger et al., 1972). Reinitiation of growth by brief proteolytic treatment of confluent cells was accompanied by a drop in the level of intracellular cAMP. Furthermore, this growth stimulation by protease was inhibited by the addition of dibutyryl cAMP (dbcAMP) (Burger et al., 1972). On the other hand, virally transformed fibroblasts exhibited uncontrolled proliferation and this altered state was linked to their inability to accumulate normal amounts of cAMP (Sheppard, 1972). More recently, cGMP has also been shown to be involved in cell proliferation. Rudland et al. (1974) observed that resting normal fibroblasts have a high cAMP/cGMP ratio whereas growing, or serum activated, cells have a low cAMP/cGMP ratio. On the other hand, transformed fibroblasts have relatively constant cAMP and cGMP levels whether during growth or at confluence.

In addition to regulating cell proliferation, cAMP has also been shown to be involved in cell differentiation. Thus, the addition of exogenous cAMP to cell cultures often results in the expression of a specialized function characteristic of the cell type. For example, the addition of cAMP, or agents which elevate cAMP levels, to immature T lymphocytes from the spleen or bone marrow of nu/nu mice induces the differentiation of these cells into mature and functional T lymphocytes (Scheid et al., 1973). Pigment production in melanomas is induced by the addition of dbcAMP (Johnson and Pastan, 1972).

The evidence for the involvement of cAMP in lymphocyte triggering is summarized in Table 1.2. Increases in intracellular cAMP levels however are insufficient for lymphocyte triggering. Thus, prostaglandin E_1 could enhance intracellular cAMP levels in rat lymph node cells but did not induce them to go into mitosis

Table 1.2

Evidence for the involvement of cAMP in lymphocyte triggering

	Observations	References
1.	DbcAMP stimulated RNA and DNA synthesis as well as enhancing the phosphorylation of histones in a manner analogous to PHA stimulation of pig lymphocytes.	Cross and Ord, 1971.
2.	Stimulation by PHA was blocked by inhibitors of adenyl cyclase (e.g. chlorpromazine). The lymphocyte response to dbcAMP was less inhibitable by chlorpromazine.	Cross and Ord, 1971; Krishnaraj and Talwar, 1973.
3.	PHA stimulated adenyl cyclase and increased intracellular cAMP level within a few minutes.	Smith et al., 1971a; Krishnaraj and Talwar, 1973.
4.	Cyclic AMP induced rat thymocytes to go into mitosis.	MacManus et al., 1971a.
5.	Agents that raised intracellular cAMP levels (e.g. dbcAMP, AP, isoproterenol and prostaglandins) inhibited PHA-induced lymphocyte transformation. These observations were interpreted to mean that higher than optimal levels of cAMP were inhibitory.	Smith et al., 1971b.

(Novogrodsky and Katchalski, 1970). Hadden et al. (1972) reported that purified PHA lost its ability to increase intracellular cAMP levels and yet retained its mitogenic properties. This raises serious doubts as to the direct role of cAMP in mediating lymphocyte transformation.

The involvement of cGMP in lymphocyte triggering is suggested by the studies of Hadden et al. (1972). They observed that Con A or PHA, when added to human blood lymphocytes at mitogenic concentrations stimulated rapid increases in cellular cGMP levels in these cells. More recently, LPS has also been shown to induce cGMP accumulation in spleen cells from athymic mice (Watson, 1975). The addition of exogenous cGMP could also stimulate DNA synthesis in normal (Weinstein et al., 1974) and T-depleted spleen cultures (Watson, 1975). However, elevated cGMP alone seems to be insufficient for lymphocyte triggering since carbamylcholine (an agent which raises intracellular cGMP levels) is not a B or a T cell mitogen (Greaves et al., 1974; Watson, 1975).

K. Rationale for this Work

Cyclic AMP has been shown by several groups to affect the induction of primary immune responses. These observations are described in the Introduction to Chapter III, and form the starting point for the experimental work to be described.

Since the expression of immunity involves the proliferation of an antigen-reactive clone, the manner in which lymphocyte proliferation is regulated is of primary importance. As discussed above, cAMP and cGMP appear to play a role in regulating cell proliferation.

Thus, cyclic nucleotides are potentially useful tools to study how the proliferation of antigen-stimulated cells is normally regulated. An important feature of the immune system is the participation of multiple cell types in the initiation of an primary immune response. One may expect cAMP to have different effects on different cells of the immune system. This property of cAMP makes it useful for studying the interactions between cell types. These studies are dealt with in detail in Chapters III, IV and V.

CHAPTER II

GENRAL MATERIALS AND METHODS

A. Materials

Materials	Source
Chemicals:	
1. General laboratory chemicals and reagents.	Fisher Scientific Company, Montreal.
2. Adenosine-3',5'-cyclic monophosphate; $N^6,O^{2'}$-dibutyryl-adenosine-3',5'-cyclic monophosphate; aminophylline and imidazole.	Schwarz/Mann, Orangeburg, New York.
3. Guanosine-3',5'-cyclic monophosphate; $N^2,O^{2'}$-dibutyryl-guanosine-3',5'-cyclic monophosphate; 8-bromo-guanosine-3',5'-cyclic monophosphate; carbamylcholine chloride and papaverine.	Sigma Chemical Company, St Louis, Mo.
4. Adenosine-5'-monophosphate; adenosine and guanosine.	Raylo Chemicals Limited, 8045 Argyll Road, Edmonton, Alberta.
5. 2-mercaptoethanol.	Eastman Kodak Company, Rochester, New York.
6. Chlorhexidine acetate	Ayerst Laboratory, Montreal, P. Q.
Media ingredients:	
1. Minimum essential medium (MEM); fetal calf serum (FCS); essential amino acids (50x); non-essential amino acids (100x); glutamine (lyophilized).	Grand Island Biological Company, Grand Island, New York.
2. Bacto heart infusion broth and Noble agar.	DIFCO Laboratory. Detroit, Michigan.

Animals. Male and female mice of the CBA/J, CBA/CaJ, or DBA/2J strain were purchased from The Jackson Laboratory, Bar Harbor, Maine at 8 weeks of age. The mice were maintained on Purina mouse chow and water in the Health Science Animal Center, University of Alberta and used at 2 to 6 months of age.

Antigens. Sheep erythrocytes (SRBC) were collected aseptically in Alsever's solution and stored as a 20% v/v solution at 4^{0}. They were washed (1500g, 7 min) twice in normal saline and once more in medium before being used for in vitro stimulation. Polymerized flagellin (POL) from Salmonella adelaide (strain SW 1338, H antigen fg, O antigen 35) was prepared according to the method of Ada et al. (1964). It was stored at -20^{0} at 2 to 3 mg per ml in sterile double-distilled water (ddH_2O).

B. Methods

Preparation of tissue culture medium. To a mixture containing 9.8g of MEM, 3.5g $NaHCO_3$, 10 ml of sodium pyruvate (100x), 10,000 units of penicillin and 10,000 µg of streptomycin, ddH_2O was added to 950 ml. The pH of the medium was adjusted to about 7.2 by passage of CO_2. The medium was sterilized by means of millipore filtration. 100 ml of heat inactivated FCS (30 min, 56^{0}) was then added. Where present, the medium contained $5x10^{-5}$ M 2-mercapto-ethanol (2-Me).

Nutritional cocktail was prepared in the following manner: 9.8g of MEM were dissolved in 950 ml of ddH_2O. To 35 ml of this solution were added 5 ml of essential amino acid (50x), 2.5 ml of nonessential amino acid (100x), 2.5 ml of 200 mM glutamine and

7.5 ml of a solution containing 7.5% w/v $NaHCO_3$ and 6.7% w/v dextrose. FCS was then added to give a final concentration of 30% v/v. The nutritional cocktail was stored at -20^0 until use.

Solutions. Puck's saline was prepared by dissolving 8.0g NaCl, 0.4g KCl, 1.0g dextrose, 0.35g $NaHCO_3$ and 0.10 g of phenol red in 1 liter of ddH_2O. The ingredients for the Mishell-Dutton balanced salts solution (BSS) were: 1.0g dextrose, 0.06g KH_2PO_4, 0.36g $Na_2HPO_4 \cdot 7H_2O$, 0.40g KCl, 8.0g NaCl, 0.19g $CaCl_2 \cdot 2H_2O$, 0.20g $MgCl_2 \cdot 6H_2O$, 0.20g $MgSO_4 \cdot 7H_2O$ and 0.10g phenol red per liter. Phosphate buffered saline (PBS) contained 8.0g NaCl, 0.20g KCl, 1.15g Na_2HPO_4 in 1 liter of ddH_2O.

Preparation of spleen cells. Aseptic techniques were used throughout. Mice of the same strain, age and sex were killed by cervical dislocation. Not more than five spleens were pooled in 10 ml of Puck's saline, acidified by passage of CO_2. The spleens were minced with fine scissors and gently teased through a stainless steel mesh. The cell suspension was spun in a 17x100 mm polystyrene (Falcon) tube at 750g for 7.5 min. The pellet was resuspended in two volumes of Puck's saline (pH = 8.0), allowing a few seconds for the dead cells to clump. The tube was then filled with medium and spun at 750g for 7.5 min. The cell pellet was finally resuspended in medium. The viability of the single spleen cell suspension was determined by counting in a hemacytometer using 0.1% w/v eosin Y (Hanks and Wallace, 1958) in PBS and 10% FCS to stain dead cells. The viability of spleen cells using this procedure was usually about 90%.

Culture systems. The culture systems of Diener-Armstrong (1969) and Mishell-Dutton (1967) were used throughout this study.

In the Diener-Armstrong system, usually $2x10^7$ viable spleen cells plus 0.02% v/v SRBC and/or 250 ng POL per ml in a total volume of 1 ml were added to a 1.1 cm diameter glass tube with its end covered by a piece of dialysis membrane. The tube was then placed in a conical flask containing 50 ml of medium, care being taken to ensure that the liquid levels on both sides were the same. Each experimental group was usually done in quadruplicate. The flasks were incubated for 4 days at 37^o in a gas mixture of 10% CO_2 in air. No daily feeding of cultures was necessary using this method.

In the Mishell-Dutton system each 35x10 mm tissue culture dish (Lux Scientific Corporation, Thousand Oaks, Calif.) contained $1x10^7$ viable spleen cells plus 0.02% v/v SRBC and/or 100 ng POL per ml and $5x10^{-5}$ M 2-Me (Click et al., 1972) in a total volume of 1.0 ml. The dishes were placed on a Bellco rocking platform and rocked at 6 to 7 oscillations per min. Each experimental group was usually done in quadruplicate. The cultures were incubated for 4 days at 37^o in a gas mixture of 10% CO_2 in air. Each culture was fed daily with 0.1 ml of nutritional cocktail.

At the end of the culture period the cells were harvested in Mishell-Dutton BSS and washed twice in the same solution before assay. Each culture was resuspended in a total volume of 0.5 ml in Mishell-Dutton BSS.

Assays. Anti-SRBC AFC were assayed by the method of Cunningham and Szenberg (1968). The assay mixture (total volume =

0.17 ml) contained 3% v/v SRBC, 0.05% v/v guinea pig serum as a source of complement and an appropriate number of immune cells. Formation of plaques was allowed to occur for 1 hr at 37°.

Anti-POL AFC were enumerated by the bacterial cytoadherence assay of Diener (1968) with the following modifications. S. derby (strain 721, H antigen fg) was grown overnight in 5 ml of nutrient broth at 25°. This procedure prevented the formation of long chains of bacteria which would artificially increase the number of colonies scored, and thus the number of AFC. The bacterial suspension was neutralized with NaOH before use and the cells were incubated for a further 15 min at room temperature before being plated out in 1 ml of agar (2.5% heart infusion broth; 1.5% w/v Noble agar). This method yielded larger colonies without affecting their number.

Results for anti-SRBC and anti-POL assays are expressed as the arithmetic mean ± standard error of the mean (SEM). Statistical calculations were done on a Wang 500 calculator.

Other methods are described in the appropriate chapters.

CHAPTER III

EFFECTS OF CYCLIC AMP AND CYCLIC GMP ON IMMUNE RESPONSES

A. Introduction

The earliest evidence suggesting that cAMP may play a role in immune responses was indirect. The double-stranded hybrid of polyriboadenylic and polyribouridylic acids (poly A:U) can enhance the AFC response to SRBC both *in vivo* (Ishizuka *et al.*, 1970; Braun and Ishizuka, 1971a,b) and *in vitro* (Braun and Ishizuka, 1971a; Ishizuka *et al.*, 1971; Jaroslow and Ortiz-Ortiz, 1972). This action of poly A:U was potentiated by the cAMP phosphodiesterase inhibitor, theophylline (Braun and Ishizuka, 1971a,b; Ishizuka *et al.*, 1971). Although poly A:U itself has little demonstrable effect on adenyl cyclase or intracellular cAMP levels (Winchurch *et al.*, 1971; Mozes *et al.*, 1974), Braun and coworkers have suggested that it acts synergistically with antigen to increase intracellular cAMP levels, perhaps only in antigen-recognizing cells. Higher, but non-toxic amounts of poly A:U, inhibited immune responses to SRBC (Braun and Ishizuka, 1971a). These observations led Braun and coworkers to suggest that an antigen, on reacting with lymphocytes, stimulates the biosynthesis of endogenous cAMP, and that this rise in cAMP level constitutes an activation signal.

More direct evidence for the involvement of cAMP in lymphocyte activation comes from the studies of Plescia *et al.* (1975). They showed that intravenous injection of SRBC into mice resulted in a biphasic change in the level of splenic cAMP;

a two to three fold increase within two minutes, followed by a drop to below normal levels during days two to five. This change in the splenic level of cAMP occurred in response to heterologous erythrocytes, but not to autologous (nonimmunogenic) erythrocytes or nonimmunogenic carbon particles. Furthermore, poly A:U was immunoenhancing if given with SRBC when the cAMP level was rising, and immunosuppressive if given when the cAMP level was decreasing.

On the other hand, Watson et al. (1973) presented data suggesting to them that elevated intracellular cAMP level is a mediator of immune paralysis, or tolerance. Addition of dbcAMP to mouse spleen cultures at early times reduced the number of AFC to SRBC. The inhibition by dbcAMP could be partially overcome by cGMP. In nu/nu spleen cultures, daily feeding of cGMP could also greatly increase the response to SRBC, a TD antigen (Watson et al., 1973). These observations were interpreted along the lines of the two signal model (p. 17), i.e. the paralytic signal delivered to B cells by antigen is cAMP and the inductive signal (normally delivered by T cells) takes the form of cGMP.

Further experimentation is therefore required to resolve this controversy regarding the role of cyclic nucleotides, especially cAMP, in the initiation of an immune response. The data to be presented will show that increasing intracellular cAMP levels during the first 12 hr of primary humoral or cell-mediated immune responses increases their magnitude. Under the same conditions, cGMP has no direct effect but inhibits cAMP-mediated stimulation. The effects of cAMP in the humoral response are antigen-dependent and antigen-specific.

B. Materials and Methods

Materials

The mastocytoma line P815 (H-2^d) was maintained by weekly passage through DBA/2J hosts (H-2^d). Sodium-^{51}Cr-chromate was purchased from New England Nuclear Company.

Methods

Treatment with pharmacological agents. Pretreatment of cells with the agents was carried out in Falcon Petri dishes at $5x10^4$ cells per mm^2 and $1x10^7$ cells per ml. Agents were removed by washing the cells three times with medium at 4^o. Since some of the test agents used were cytotoxic at higher concentrations, the number of cells recovered in control and test groups after a 12 hr incubation could differ by 20 to 50%, depending on culture conditions. This difference in cell number was not compensated for when the cells were recultured in either the Mishell-Dutton or the Diener-Armstrong system, i.e. each flask contained the viable cells from $2x10^7$ cells, or each tissue culture dish contained the viable cells from $1x10^7$ cells, added at 0 time.

Generation of cytotoxic lymphocytes. Cytotoxic lymphocytes to DBA/2J (H-2^d) were generated in a one-way mixed lymphocyte culture by incubating $1.5x10^7$ viable CBA/J (H-2^k) spleen cells with $2x10^6$ irradiated (1000 rads whole body irradiation from a ^{137}Cs source: Gamma Cell 40, Atomic Energy of Canada Ltd.) DBA/2J spleen cells in Diener-Armstrong flasks. Five days later the cells were harvested and washed twice with medium before assaying for cytolytic activity against ^{51}Cr-labelled P815 mastocytes.

Assay for cytotoxic lymphocytes. The ability of sensitized

CBA/J lymphocytes to kill P815 mastocytes was assayed according to Brunner et al. (1968). Cytotoxic lymphocyte to target ratios of 40, 20, 10, and 5 to 1 were used. Each ratio was done in duplicate with 3×10^4 ^{51}Cr-labelled target cells in 12x75 mm polystyrene (Falcon) tubes in a total volume of 0.5 ml. Incubation was at 37^o. At the end of 5 hr, 1 ml of 0.2% v/v SRBC was added and the tubes were spun at 750g for 10 min. The top 1 ml supernatant and 1/2 ml supernatant plus pellet were counted in a gamma counter. Per cent specific lysis were calculated from the equation:

$$\% \text{ specific lysis} = \frac{\% \text{ release of test group} - \% \text{ release of bkg}}{\% \text{ maximum release} - \% \text{ release of bkg}} \times 100$$

$$\text{where } \% \text{ release} = \frac{\text{Supernatant counts}}{\text{Supernatant counts + pellet counts}} \times 100$$

and bkg = background. Background release was determined in the same manner as test groups except that the spleen cells were obtained from unimmunized cultures. Maximum ^{51}Cr release was achieved by freezing and thawing 3×10^4 labelled target cells three times and then incubating the cells at 37^o for 5 hr.

The results were expressed as lytic units (MacDonald et al., 1974) per culture. One lytic unit is defined as the number of cells required to lyse 50% of 1×10^4 ^{51}Cr-labelled P815 target cells under the conditions described above. Lytic units per culture is therefore a relative measure of the frequency of cytotoxic lymphocytes in various cell populations.

C. Results

Long term effects of cAMP and cGMP on the AFC response to SRBC and POL

Cyclic AMP, cGMP, and agents that enhance the intracellular levels of these two cyclic nucleotides were tested for their abilities to affect antibody responses. Normal CBA/J spleen cells were incubated with various concentrations of these agents in the presence of SRBC and POL for the entire length of the culture period. The results are shown in Table 3.1. DbcAMP and aminophylline (AP) were stimulatory (2 to 3 fold) in the 10^{-6} to 10^{-4} M range, peaking at 10^{-4} M. Both responses were completely inhibited by these agents at 10^{-3} M. Lower concentrations of papaverine, a more potent inhibitor of the cAMP phosphodiesterase (Sheppard et al., 1972), were required to achieve the same effects. Cyclic AMP could also inhibit the anti-SRBC response at 10^{-3} M but did not stimulate the response at lower concentrations. In all cases inhibition by these agents at high concentrations was directly correlated with their cytotoxic effects.

On the other hand, cGMP and its dibutyrated or 8-bromo-derivatives had little or no effect on the AFC response to SRBC or POL over a concentration range of 10^{-7} to 10^{-3} M; the cholinergic agent, carbachol, also had no effect (Table 3.1). These observations are in agreement with those reported by other workers (Watson et al., 1973; Watson, 1975).

Early effects of cAMP and cGMP on humoral responses

Since transient increases in endogenous cyclic nucleotides levels have been observed following the addition of mitogens or SRBC

Table 3,1

Long term effects of cAMP, cGMP, and related compounds on AFC responses

Agents[a]	Concentration in Molarity						AFC/culture[d] to
	-	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	
Cyclic AMP[b]	5337	n.d.	n.d.	5350	5823	100	SRBC
DbcAMP[b]	5187	"	6037	9300	10437	100	SRBC
	3775	"	5750	7100	9700	100	POL
Aminophylline[b]	5187	"	6075	8233	10555	100	SRBC
	3775	"	3900	6600	10050	100	POL
Papaverine[b]	3050	3612	5125	1691	100	n.d.	SRBC
	2260	3410	5365	2135	100	"	POL
Cyclic GMP[c]	2250	2266	3141	2691	2650	2925	SRBC
	1368	1266	2040	2473	1633	1320	POL
DbcGMP[c]	2250	3008	2300	2568	2825	1850	SRBC
	1386	1580	1633	1800	2373	1920	POL
8-Br-cGMP[c]	1383	1800	1616	1366	1383	1866	SRBC
Carbachol[b]	7341	6116	6083	6091	7291	n.d.	SRBC
	6240	8393	8293	5086	7526	"	POL

[a] These agents were present throughout the 4-day culture period.

[b] Diener-Armstrong culture system (each culture contained 2×10^7 cells + 0.01% v/v SRBC + 250 ng POL/ml).

[c] Mishell-Dutton culture system (each culture contained 1×10^7 cells + 0.01% v/v SRBC + 100 ng POL/ml).

[d] Arithmetic mean of four cultures. The standard error of the mean (not shown) was in most cases less than 10% of the mean. n.d. = not determined.

to lymphocytes it was thought that agents that would elevate intracellular cyclic nucleotides levels might be immunoenhancing when present only at early times in antigen-stimulated cultures. This was tested by treating antigen-stimulated spleen cells with various concentrations of the appropriate agent for the first 12 hr of a 108 hr incubation period. The effects of "pulsing" the cultures with cAMP, its breakdown products (5'-AMP and adenosine), dbcAMP, AP, or butyric acid on the AFC response to SRBC are shown in Table 3.2. DbcAMP and AP were most effective (2.6 to 4.3 fold) in stimulating the AFC response at 10^{-3} M. At this concentration dbcAMP and AP have been reported to inhibit the cAMP phosphodiesterase activity in fetal rat calvaria, leading to a 2 to 3 fold increase in intracellular cAMP level (Heersche et al., 1971); AP can also increase lymphocyte cAMP level (Smith et al., 1971a). Lower concentrations were less effective or ineffective. Adenosine or butyric acid gave about a 2 fold enhancement of the response at 10^{-3} M; whereas cAMP or 5'-AMP were completely ineffective in enhancing the anti-SRBC response. The stimulatory effects of adenosine and butyric acid can also be attributed to cAMP since these agents have been shown to increase cAMP levels in cytotoxic lymphocytes (Wolberg et al., 1975) and neuroblastoma cells (Sheppard and Prasad, 1973), Similar results were obtained in the Diener-Armstrong or the Mishell-Dutton culture systems.

On the other hand, cGMP, dbcGMP, 8-Br-cGMP, or carbamylcholine chloride (carbachol) did not have any effect on the anti-SRBC response under similar experimental conditions (Table 3.2). These studies indicate that cAMP, but not cGMP, is immunoenhancing

Table 3.2

Early effects of cAMP, cGMP, and related compounds on the AFC response to SRBC

Agents	Concentrations during the 0-12 hr incubation period				Culture systems
	-	10^{-5} M	10^{-4} M	10^{-3} M	
Cyclic AMP	6050 ± 304[a]	n.d.	6200 ± 730	4500 ± 320	Diener-Armstrong
	1300 ± 189	1350 ± 81	1033 ± 158	1483 ± 44	Mishell-Dutton
DbcAMP	2850 ± 70	2790 ± 300	3810 ± 500	12200 ± 1600[b]	Diener-Armstrong
	1300 ± 189	1650 ± 526	2650 ± 579	5200 ± 407[b]	Mishell-Dutton
Aminophylline	2093 ± 319	2340 ± 290	1920 ± 74	5453 ± 808[b]	Diener-Armstrong
	560 ± 52	700 ± 52	920 ± 17[b]	1900 ± 160[b]	Mishell-Dutton
Adenosine	1300 ± 189	1383 ± 235	1333 ± 142	2416 ± 284[c]	Mishell-Dutton
5'-AMP	1300 ± 189	1350 ± 152	1883 ± 208	1983 ± 208	Mishell-Dutton
Butyric acid	1300 ± 189	1183 ± 334	1950 ± 152	2850 ± 50[b]	Mishell-Dutton
Cyclic GMP	1300 ± 189	1050 ± 132	1160 ± 280	1116 ± 169	Mishell-Dutton
DbcGMP	1300 ± 189	1150 ± 104	1066 ± 60	1633 ± 101	Mishell-Dutton
8-Br-cGMP	1300 ± 189	1133 ± 101	1150 ± 144	1050 ± 246	Mishell-Dutton
Carbachol	560 ± 52	460 ± 54	520 ± 173	440 ± 90	Mishell-Dutton

Spleen cells were incubated with various concentrations of the indicated agent and 0.01% v/v SRBC for 12 hr in Falcon Petri dishes as described in the Methods section. At the end of the 12 hr incubation period, the agents were removed by washing and the cells recultured with SRBC only, in either the Diener-Armstrong or the Mishell-Dutton culture system in quadruplicate. All cultures were assayed at 108 hr. [a]Arithmetic mean ± SEM; [b]$P < 0.05$; [c]$0.05 < P < 0.1$. The rest of the values were not significantly different from the controls ($P > 0.1$).

during the early phase of immune responses.

The length of the 10^{-3} M dbcAMP pulse required for maximal stimulatory effects was next determined. It is clear from Table 3.3 that the best stimulation was obtained with a 0-12 hr pulse. It should be noted that at the concentration used, dbcAMP was cytotoxic, as indicated by the smaller number of viable cells recovered at the end of the pulse compared to control cultures. Despite this, an increase in the number of AFC was obtained. This was true even when dbcAMP was present for up to 22 hr.

Stimulation of immune responses by cAMP is dependent on "good" culture conditions

Since other workers have concluded that cAMP is immunosuppressive (Watson et al., 1973; Bösing-Schneider and Kolb, 1973) the conditions under which cAMP-enhancing agents could inhibit the anti-SRBC response at early times were next investigated. The cells were pulsed with these agents either in Petri dishes as was normally done or in 17x100 mm polystyrene (Falcon) tubes. It was found that when the cells were preincubated in Falcon tubes, the cell pellet formed at the bottom of the tube was much more susceptible to killing by AP than when the cells were incubated in Petri dishes. As a result the response of cells treated with AP and antigen for as little as 6 hr in tubes gave a much lower response than control cultures (Table 3.4). The immunosuppressive actions of cAMP at early times could therefore be due to general cytotoxic actions.

It has become common practice for some workers to incubate the spleen cells overnight in tissue culture medium before adding

Table 3.3

A 12 hr "pulse" of 10^{-3} M dbcAMP is required for maximum enhancement of the anti-SRBC response

Duration of DbcAMP pulse in hr	% of starting cells recovered at end of pulse		PFC/culture at 108 hr (mean ± SEM)		Cells recovered at 108 hr ($x10^{-6}$/culture)		Stimulation index
	Normal	+DbcAMP	Normal	+DbcAMP	Normal	+DbcAMP	
0-9.5	92	71	9400 ± 458	16900 ± 1300	6.8	7.7	1.8
0-12	94	65	8100 ± 755	24033 ± 1910	7.7	7.7	2.8
0-16	77	52	11766 ± 285	23966 + 1340	10.4	10.4	2.0
0-22	78	35	6833 ± 384	15066 ± 920	9.3	6.6	2.2
0-31.5	67	23	7900 ± 360	6166 ± 900	8.4	2.0	0.78

Spleen cells were incubated with 0.01% v/v SRBC and 10^{-3} M dbcAMP in Petri dishes for the times indicated. At the end of the pulse the cells were recultured with SRBC only, in quadruplicate in the Diener-Armstrong system. Each flask contained the cells from $3x10^7$ viable spleen cells added at 0 time.

Table 3.4

Stimulatory effects of dbcAMP or aminophylline is dependent on "good" culture conditions

A. First incubation carried out in Petri dishes.

Additives during 0-6 hr incubation	Anti-SRBC PFC/culture (mean ± SEM)	Anti-POL AFC/culture (mean ± SEM)
SRBC + POL	8631 ± 346	5587 ± 514
" + " + 10^{-3} M dbcAMP	11681 ± 800	6925 ± 976
" + " + 10^{-4} M "	11850 ± 336	5762 ± 667
" + " + 10^{-3} M AP	8587 ± 683	7887 ± 1326
" + " + 10^{-4} M "	10193 ± 610	5325 ± 594

B. First incubation carried out in (Falcon) tubes.

Additives during 0-6 hr incubation	Anti-SRBC PFC/culture (mean ± SEM)	Anti-POL AFC/culture (mean ± SEM)
SRBC + POL	6137 ± 519	4337 ± 532
" + " + 10^{-3} M AP	612 ± 249	1150 ± 176*

The first incubation was carried out either in Falcon Petri dishes ($8x10^7$ cells per dish in a total volume of 4 ml) or in Falcon tubes ($8x10^7$ cells per tube in a total volume of 4 ml). The concentrations of antigen used were 0.01% v/v SRBC and 250 ng POL/ml. At the end of 6 hr the cells were washed twice with medium and recultured with both antigens in quadruplicate in the Diener-Armstrong system. The cultures were assayed at 96 hr.

antigen. It was therefore decided to test if dbcAMP could stimulate the response to SRBC or POL under these conditions. The results in Table 3.5 show that although the number of cells recovered from the preincubated group after the 12 hr treatment was less than that of fresh cells, the surviving cells from the dbcAMP treated group could still give an increase in the number of AFC to either SRBC or POL over control values. Thus, dbcAMP could stimulate both fresh spleen cells and spleen cells that have been incubated overnight in the absence of antigen.

The actions of cAMP phosphodiesterase inhibitors at later times of the immune response

Cyclic AMP has an antimitotic effect on the growth of fibroblasts (Burger et al., 1972). Also, since the change in cellular levels of cAMP following stimulation of lymphocytes with mitogen (Smith et al., 1971a) or antigen (Plescia et al., 1975) is biphasic, it was predicted that a high cAMP level would be immunosuppressive once antigen-stimulated cells are proliferating (i.e. after 24 hr). Therefore a 5-day culture period was divided into five 24 hr intervals. 10^{-3} M AP was added to each of these 24 hr intervals and removed by washing with medium at the appropriate times. The results are shown in Fig. 3.1. Only treatment with AP during the first 24 hr period stimulated the response. When AP was added later strong inhibition of the anti-SRBC response was observed, being maximal at 48 to 72 hr and least at 96 to 120 hr. Similar observations were obtained when the cultures were treated with 10^{-3} M dbcAMP or when the anti-POL response was measured. The proliferation

Table 3.5

Effects of dbcAMP on the AFC responses of spleen cells that had been preincubated overnight in medium

Additives during 0-12 hr	% of starting cells recovered at 12 hr		Anti-SRBC (PFC/culture) (mean ± SEM)		Anti-POL (AFC/culture) (mean ± SEM)	
	Fresh	Preincubated	Fresh	Preincubated	Fresh	Preincubated
SRBC	97	90	1360 ± 138	920 ± 84	n.d.	n.d.
SRBC + dbcAMP	73	51	4900 ± 538	2853 ± 332	"	"
POL	96	83	n.d.	n.d.	2430 ± 248	2170 ± 293
POL + dbcAMP	71	50	"	"	4665 ± 365	3550 ± 182

Fresh spleen cells or spleen cells that had been preincubated for 12 hr in medium were treated with or without 10^{-3} M dbcAMP in the presence of 0.02% v/v SRBC or 100 ng POL/ml. After removal of the dbcAMP at 12 hr, the cells were cultured with antigen in the Mishell-Dutton culture system in quadruplicate. Each culture dish contained the equivalent of $1x10^7$ viable cells added at 0 time. All cultures were assayed at 108 hr.

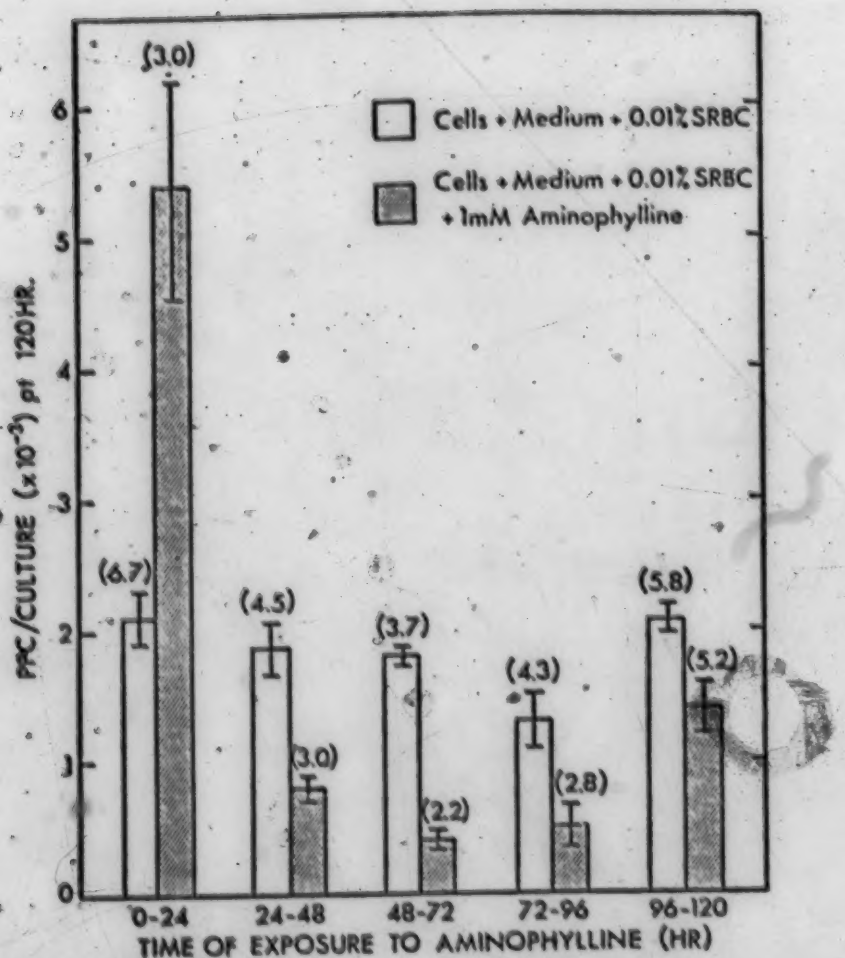


Fig. 3.1. Effects of adding AP at different times of the culture period on the anti-SRBC response. All cultures were set up in triplicate in the Diener-Armstrong system. Each flask contained 2×10^7 viable spleen cells plus 0.01% v/v SRBC. At the times indicated, 10^{-3} M AP was added. Removal of the AP was achieved by washing the cells twice with 10 ml of medium. The control cells were in each case handled the same way as experimental cultures. After washing the cells were recultured with fresh medium in a fresh set of culture flasks without readjustment of cell densities. Number in parentheses indicate the number of viable cells, in millions, recovered per culture at 120 hr.

of antigen-stimulated lymphocytes thus seems to be be inhibited by high cAMP levels.

Reversal of the stimulatory effects of dbcAMP

If elevated cAMP levels stimulate immune induction, then prevention of this elevation should counteract the stimulation. Cyclic GMP and imidazole have been reported to stimulate the intracellular hydrolysis of cAMP (Beavo et al., 1970; Watson, 1974). Consequently, these compounds were tested for their ability to reverse the effects of dbcAMP. The results are shown in Table 3.6. Cyclic GMP or imidazole gave about a two fold stimulation of the anti-SRBC response when present from 0-24 hr at 10^{-3} M and 10^{-4} M respectively. When added simultaneously with dbcAMP to cultures, these agents considerably reduced the stimulatory effects caused by dbcAMP alone (from 4 fold to 2 fold). Con A, which has been reported to elevate cGMP levels in lymphocytes (Hadden et al., 1972), could also counteract the stimulation by dbcAMP (from 3 fold to normal levels). Since Con A has also been shown to affect the functions of T cells (Dutton, 1972), the inhibition of the dbcAMP effect could be due to factors other than cGMP. Addition of imidazole to cultures subsequent to pretreatment with dbcAMP also reduced the stimulatory effect of dbcAMP (Table 3.6).

The dbcAMP effect is antigen-dependent and antigen-specific

The data in Table 3.7 show that the presence of antigen is required for dbcAMP to exert its enhancing effect on the immune response. This is true for the AFC response to either SRBC or POL. The effect is also antigen-specific, i.e. if dbcAMP is added

Table 3.6

The stimulatory effect of dbcAMP is diminished by simultaneous incubation with cGMP, imidazole, or Con A

Expt.	Additives during the 0-24 hr incubation	Additives during the 24-120 hr incubation	Anti-SRBC PFC/culture (mean ± SEM)
I	SRBC	SRBC	3766 ± 450
	SRBC + dbcAMP	SRBC	15000 ± 1030
	SRBC + cGMP	SRBC	7541 ± 610
	SRBC + imidazole	SRBC	6300 ± 470
	SRBC + cGMP + dbcAMP	SRBC	9225 ± 630
	SRBC + imidazole + dbcAMP	SRBC	7441 ± 600
	SRBC	SRBC + imidazole	4150 ± 655
	SRBC + dbcAMP	SRBC + imidazole	6591 ± 1340

II	Additives during the 0-12 hr incubation	Anti-SRBC PFC/culture (mean ± SEM)
	SRBC	6366 ± 168
	SRBC + dbcAMP	20333 ± 1230
	SRBC + Con A	6900 ± 408
	SRBC + Con A + dbcAMP	7133 ± 1145

The spleen cells were incubated with 0.01% v/v SRBC ± test agent(s) in Petri dishes for 0-24 hr or 0-12 hr as indicated. At the end of the incubation the agents were removed and the cells recultured in the Diener-Armstrong system. Cultures in experiment I were assayed at 120 hr and cultures in experiment II were assayed at 108 hr. Concentrations of the agents used: dbcAMP, 10^{-3} M; cGMP, 10^{-3} M; imidazole, 10^{-4} M; and Con A, 5 μg/ml.

Table 3.7

The stimulatory effect of dbcAMP is both antigen-dependent and antigen-specific

Additives during the 0-12 hr incubation	Additives during the 12-108 hr incubation	PFC/culture to SRBC (mean ± SEM)	AFC/culture to POL (mean ± SEM)
Medium only	SRBC	2665 ± 485	n.d.
Medium + 10^{-3} M dbcAMP	SRBC	3275 ± 716	"
Medium only	POL	n.d.	1880 ± 81
Medium + 10^{-3} M dbcAMP	POL	"	2453 ± 181
SRBC	SRBC	2791 ± 374	n.d.
SRBC + 10^{-3} M dbcAMP	SRBC	9516 ± 2060	"
SRBC	SRBC + POL	4525 ± 683	2573 ± 88
SRBC + 10^{-3} M dbcAMP	SRBC + POL	14733 ± 2080	2426 ± 169
POL	POL	n.d.	2980 ± 652
POL + 10^{-3} M dbcAMP	POL	"	7680 ± 880
SRBC + POL	SRBC + POL	6916 ± 520	3960 ± 620
SRBC + POL + 10^{-3} M dbcAMP	SRBC + POL	11066 ± 1340	8320 ± 191
POL	SRBC + POL	4366 ± 670	2973 ± 810
POL + 10^{-3} M dbcAMP	SRBC + POL	2908 ± 240	9733 ± 520

Spleen cells were incubated with 0.01% v/v SRBC and/or 250 ng POL/ml with or without 10^{-3} M dbcAMP for 12 hr in Petri dishes. The cells were then recultured in the Diener-Armstrong system with the specified antigen(s). All cultures were assayed at 108 hr.

with antigen A for 12 hr, then removed, and the cells recultured with antigen A plus antigen B, only the response to antigen A is stimulated. It should also be noted that POL by itself has an enhancing effect on the anti-SRBC response (compare lines 1, 5,7, and 11). This immunoenhancing effect of POL could be due to its potential mitogenic properties (Coutinho and Möller, 1973).

Effects of cyclic nucleotides on the induction of cell-mediated immunity

The effects of agents which elevate intracellular cAMP levels on the inductive phase of cell-mediated immunity have not been described previously. To investigate this, cytotoxic lymphocytes were generated in vitro by incubating CBA/J ($H\text{-}2^k$) spleen cells with lethally irradiated DBA/2J ($H\text{-}2^d$) spleen cells in the presence or absence of dbcAMP. After 12 hr the cells were washed free of these agents and recultured for a further four and a half days before assaying for cytotoxic lymphocytes using ^{51}Cr-labelled P815 mastocytes as targets. The results in Fig. 3.2 show that optimum stimulation of the response was obtained when the CBA/J spleen cells were cultured with 10^{-4} M dbcAMP and stimulator cells. The stimulation was observed either at high or low stimulator cell dose. DbcAMP by itself did not stimulate the background response, i.e. it is dependent on the presence of antigen for stimulation. The results are expressed in terms of lytic units per culture in Table 3.8. On the other hand, dbcGMP under similar conditions did not have any significant effect on the induction of CMI (Table 3.9).

Finally it was decided to investigate if cAMP can have

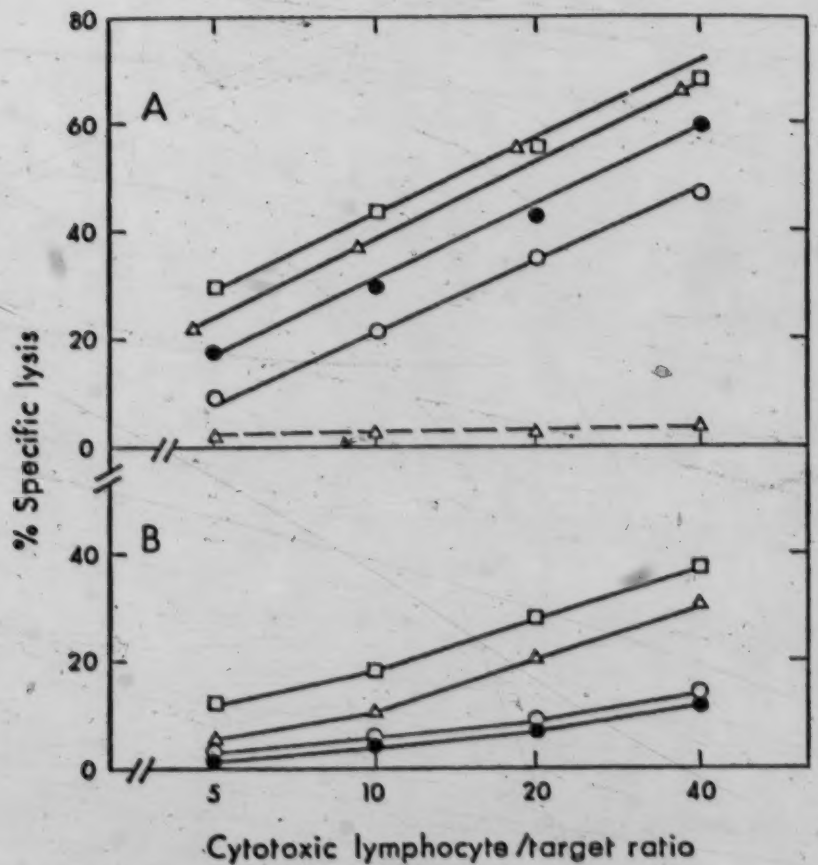


Fig. 3.2. Effects of dbcAMP on the induction of CMI. (A) Killer cells to DBA/2J were generated by incubating 1.5×10^7 CBA/J spleen cells with 2×10^6 irradiated DBA/2J spleen cells for 5 days. Various concentrations of dbcAMP were added to the cultures for the first 12 hr only. The drug was removed by washing the cells twice with medium. The cultures were assayed for cytotoxic lymphocytes according to Brunner et al. (1968), using ^{51}Cr-labelled P815 mastocytes as targets. Background supernatant counts were about 120 cpm and freeze thaw supernatant counts (100% lysis) were about 1500 cpm. (○—○), CBA/J + DBA/2J; (△---△), CBA/J + 10^{-3} M dbcAMP; (△—△), CBA/J + DBA/2J + 10^{-3} M dbcAMP; (□—□), CBA/J + DBA/2J + 10^{-4} M dbcAMP; (●—●), CBA/J + DBA/2J + 10^{-5} M dbcAMP.

(B) Conditions used were similar to (A) except that the dose of DBA/2J cells was 0.2×10^6 per culture.

Table 3.8

Early effects of dbcAMP on cell-mediated immunity

Additives at 0-12 hr	Lytic units per culture[a]	Stimulation index
2.0×10^6 DBA/2J	15.6	1.00
" + 10^{-5} M dbcAMP	20.8	1.33
" + 10^{-4} "	43.3	2.78
" + 10^{-3} "	30.4	1.95
0.2×10^6 DBA/2J	1.20	1.00
" + 10^{-5} M dbcAMP	1.39	1.16
" + 10^{-4} "	4.69	3.90
" + 10^{-3} "	3.16	2.64

The data used for this table were obtained from Fig. 3.2.

[a]A lytic unit is defined as the number of cells required to lyse 50% of 1×10^4 labelled target cells under the conditions described in "Methods".

Table 3.9

Early effects of dbcGMP on cell-mediated immunity

Expt.	Additives at 0-12 hr	Lytic units per culture	Stimulation index
I	$2x10^6$ DBA/2J	30.6	1.00
	" + 10^{-5} M dbcGMP	25.9	0.85
	" + 10^{-4} "	25.8	0.84
	" + 10^{-3} "	19.8	0.65
II	$2x10^6$ DBA/2J	121.0	1.00
	" + 10^{-4} M dbcGMP	81.9	0.68
	" + 10^{-3} "	71.4	0.59
III	$2x10^6$ DBA/2J	48.4	1.00
	" + 10^{-3} M dbcGMP	65.7	1.36

The conditions used for this experiment were similar to those described in Fig. 3.2.

different activities on T cells from mice of different ages. The results are shown in Table 3.10. There is a small increase in the capacity of spleen cells to generate cytoxic lymphocytes with increasing age of the mice. Pulsing with 10^{-4} M dbcAMP in the presence of stimulator cells increased the number of cytotoxic lymphocytes in all the cultures, being maximal when the spleen cells were obtained from 84 day-old mice.

D. Discussion

Agents that increase intracellular cAMP levels (dbcAMP, AP, adenosine and butyric acid) can enhance the AFC response of mouse spleen cultures to SRBC and POL when added at 10^{-3} M during the 0 to 12 hr period of the culture. It is not surprising that cAMP itself has no demonstrable effect since it is known to have a short half-life in medium and does not enter cells readily (MacManus et al., 1971b). The enhancing effect of dbcAMP cannot be attributed to butyric acid alone since the stimulation obtained by dbcAMP is greater than that of butyric acid, and the butyric acid residues are unlikely to be quantitatively hydrolysed.

The lack of direct effects with cGMP and related compounds (0-12hr) are unlikely to be due to their instabilities since exogenous cGMP (or imidazole), when added together with dbcAMP, could counteract the stimulatory effects of dbcAMP (Table 3.6). Moreover, when present from 0-24 hr they stimulated the AFC response to SRBC by about two fold (Table 3.6). It is conceivable that the addition of cGMP or imidazole to the cultures for up to 24 hr may aid antigen-stimulated cells already in the G1 phase of the cell cycle to proceed more readily into S phase since proliferating cells

Table 3.10

Effects of dbcAMP on the induction of cell-mediated immunity in spleen cell cultures from mice of different ages

Age (days)	Lytic units per culture		Stimulation index
	Normal[a]	+dbcAMP[b]	
40	0.905	2.63	2.91
84	1.54	10.5	6.82
280	2.26	9.63	4.26

[a] 1.5×10^7 CBA/J spleen cells from mice of the indicated age plus 2×10^6 irradiated DBA/2J spleen cells incubated for 5 days.

[b] same as normal except that 10^{-4} M dbcAMP was present in the cultures for the first 12 hr.

have been shown to have a low cAMP to cGMP ratio (Rudland et al., 1974).

The presence of antigen is obligatory for dbcAMP to exert its enhancing effect (Table 3.7). Cyclic AMP itself is therefore insufficient to induce an immune response but more likely, it constitutes part of the inductive signal. However, it is possible that only cells that have been triggered by antigen could be affected by dbcAMP. Thus, a more stringent test of the antigen-dependence and antigen-specificity of dbcAMP would be to add cells that have already been exposed to antigen for the same length of time, i.e. 12 hr. This experiment will be discussed in detail in Chapter IV, but briefly, the data support the conclusion that the dbcAMP effect is indeed antigen-dependent and antigen-specific. These findings are in contrast to those of Watson et al. (1973) who reported an early stimulatory effect of dbcAMP which was not antigen-dependent.

The above experiments are incompatible with the hypothesis that cAMP is a mediator of immune paralysis, or tolerance (Watson et al., 1973). The stimulatory effects of adding 10^{-3} M dbcAMP were also observed by Watson and coworkers; inhibition of the anti-SRBC response resulted only if dbcAMP was present for more than 12 hr. The studies in this Chapter show that the result of adding 10^{-3} M dbcAMP or AP for up to 24 hr always leads to an increased number of AFC. Inhibition during early times resulted only when the culture conditions were suboptimal (Table 3.4). The inhibitory effects of cAMP on the AFC response to SRBC observed by Bösing-Schneider and Kolb (1973) can also be explained by general cytotoxic properties of this agent.

Addition of AP or dbcAMP at later times of the culture period, however, inhibited the response (Fig. 3.1). This is expected since AFC arise as a result of proliferation of antigen-stimulated cells and high cAMP levels have antimitotic effects (Burger et al., 1972), probably as a result of arresting dividing cells at late G1 (Coffino et al., 1975).

DbcAMP also has a positive effect in the induction of cell-mediated immunity, and dbcGMP has no effect. DbcAMP is again dependent on the presence of stimulator cells (antigen) for enhancement of the response. Over the age range tested (40 to 280 days) the dbcAMP effect on the induction of CMI was not markedly age-dependent. This is not too surprising since spleen cells from 40 day-old mice already yielded a large number of cytotoxic lymphocytes, i.e. most of the splenic T cells were functionally mature by this age. It would be interesting to examine the effects of dbcAMP on the ability of neonatal spleen cells, which may have a higher proportion of immature T cells, to generate cytotoxic lymphocytes, since cAMP has been implicated in causing the maturation of T cells (Scheid et al., 1973). Agents that elevate intracellular cAMP levels were also reported to inhibit the cytolytic action of cytotoxic lymphocytes (Strom et al., 1972; Bourne et al., 1974; Wolberg et al., 1975) whereas agents that enhanced intracellular cGMP level could augment this activity (Strom et al., 1972, 1975).

To summarize, the data presented in this chapter are consistent with the following general conclusions: 1) a high intracellular level of cAMP during the early stages of the response stimulates both humoral and cell-mediated immune induction; 2) agents

able to prevent this rise in cAMP level (cGMP, imidazole, and perhaps Con A) reverse the stimulatory effects of dbcAMP; 3) the cAMP effect is antigen-dependent and antigen-specific; and 4) a high cAMP level at later times (24 hr or later) inhibits the immune response. It is therefore proposed that a rise in cAMP level constitutes part of the inductive signal for immune induction, the rest of the signal being provided by antigen in an as yet undetermined manner. The type of cell (B, T, macrophage) in which this phenomenon operates is the subject of the next chapter. However, the cAMP level should subsequently fall to a level consistent with that required for cell cycling. The studies of Plescia et al. (1975) and Yamamoto and Webb (1975) would support such an hypothesis, since they reported that intravenous injection of SRBC into mice could cause transient increase in overall splenic cAMP levels. Obviously, the 2 to 4 fold enhancement seen in their work could not be due specifically to antigen-stimulated B cells, since these constitute less than 0.01% of the spleen cell population. More likely, the cAMP accumulation was due to a general stimulation of the adenyl cyclase of spleen cells by an unknown mechanism.

CHAPTER IV

THE CELLULAR BASIS OF CYCLIC AMP ACTION IN HUMORAL IMMUNITY

A. Introduction

In Chapter III it was demonstrated that high cAMP levels during the inductive phase of a humoral response lead to increased numbers of AFC. Since the induction of an AFC response to SRBC, a TD antigen, requires the participation of a B cell, a T helper cell and a macrophage (p.13) the enhancement by cAMP of humoral immunity could theoretically result from one or more of the following: 1) direct stimulation of B cells by cAMP; 2) stimulation of T cell helper activities; 3) blocking of T cell regulatory activity ("suppressor" function); or 4) enhancement of macrophage function. These possibilities will be examined in this chapter. DbcAMP will also be used to investigate the cellular interactions underlying the AFC response to POL, a TI antigen.

The effects of cAMP on the cell types involved in HI are not well characterized. The few reports related to this subject indicate that in the presence of high cAMP levels, T and B cells have a very short half-life; i.e. a high intracellular cAMP level is cytotoxic for these cells (Watson et al., 1973). Other studies showed that the injection of poly A:U or dbcAMP can restore the response of neonatally thymectomized mice to SRBC (Cone and Johnson, 1971; Uzunova and Hanna, 1973). Poly A:U can also enhance the rate of allogeneic skin graft rejection in neonatally thymectomized

mice (Cone and Johnson, 1971). These studies taken together imply that poly A:U or dbcAMP can stimulate the residual T cell activity in neonatally thymectomized mice, or that they can cause the maturation of T cell precursors into functional T cells. Support for the latter possibility came from the observation of Scheid et al. (1973), where they reported that poly A:U, dbcAMP, or AP can cause the maturation of T cell precursors into functional T cells. The function of peritoneal exudate cells (having A cell activity) has also been reported to be augmented by poly A:U (Johnson and Johnson, 1971).

The studies in this chapter are consistent with the following conclusions: 1) dbcAMP does not increase the number of AFC by a direct B cell effect; 2) there is little evidence that dbcAMP directly stimulates T helper activity; 3) dbcAMP inhibits the function of macrophages; and 4) dbcAMP inhibits the function of a regulator (or suppressor) cell which is θ positive and radiosensitive.

B. Methods

T cell-depleted mice (ATXBM). These mice were a gift from Dr. Chiaki Shiozawa. The mice were thymectomized at four weeks of age (Miller, 1960). Two weeks later they were lethally irradiated with 950 rads from a ^{137}Cs source, and each mouse was reconstituted intravenously with $2x10^6$ syngeneic bone marrow cells the same day. The spleens of these mice were used for tissue culture 10 weeks after bone marrow reconstitution.

Anti-θ serum. The serum was a gift from Dr. Kwok-Choy Lee. AKR/J mice were each immunized with 10 weekly intraperitoneal injections of 10^8 CBA/J thymocytes. The first injection was accompanied by 10^9 killed Bordetella pertussis organisms. Mice were bled from the retro-orbital plexus after the seventh week and finally by heart puncture after the tenth week.

Anti-θ treatment. Spleen cells were incubated with the right dilution of anti-θ serum in medium at $2x10^7$ cells per ml for 40 min at 37^o. The cells were washed once and reincubated with agarose-absorbed guinea pig complement (Cohen and Schlesinger, 1970) diluted 6 times in medium for 40 min at 37^o. The cells were washed 3 times before use. About 30% specific lysis of normal spleen cells was achieved using this method.

SRBC-primed spleen cells. Normal mice, immunized by one intravenous injection of 0.2 ml of an 0.01% v/v suspension of SRBC 6 to 8 days before, were irradiated with 1000 rads just before their spleens were removed. The spleen cells were used as a source of primed T cells specific for SRBC. Spleen cells so prepared have been shown to be able to cooperate with B cells in the stimulation of a response to SRBC but are unable by themselves to proliferate into AFC (Kettman and Dutton, 1971).

C. Results

The AFC response to SRBC at different cell densities

Normal unprimed spleen cells were incubated with SRBC in the presence or absence of 10^{-3} M dbcAMP for 12 hr as described in Chapter III. This preincubation was performed in the absence

of 2-mercaptoethanol (2-Me). At the end of 12 hr the cells were recultured in the Mishell-Dutton system with each culture containing the equivalent of either $5x10^5$ or $1x10^7$ vaible spleen cells at 0 hr in the presence or absence of $5x10^{-5}$ M 2-Me. As indicated in Fig. 4.1, the anti-SRBC responses of both normal and dbcAMP treated cultures at low cell density ($5x10^5$ cells per culture) are markedly dependent on the presence of 2-Me. Thus, it is clear that at low cell numbers, a cell type is present in suboptimal numbers and it could be compensated for by 2-Me. Since 2-Me was shown to be able to replace adherent cell function (Chen and Hirsch, 1972), this limiting cell type is likely to be a macrophage. It should be noted that whereas 2-Me increases the response of normal cultures 9.6 fold, it enhances the response of dbcAMP treated cultures 82 fold (compare columns 1 and 3, 2 and 4, in Fig. 4.1). Looked at another way, dbcAMP actually inhibits the response when macrophage function (no 2-Me) is limiting.

On the other hand, when the cells were cultured at high cell density ($1x10^7$ cells per culture) the normal cells or dbcAMP treated cells were less dependent on 2-Me in supporting a response to SRBC (Fig. 4.1). 2-Me only enhanced the normal response by 1.4 fold and the dbcAMP treated response by 1.7 fold. In these cases macrophages are present in sufficiently high numbers to support a good anti-SRBC response even in the absence of 2-Me. The anti-SRBC responses were normalized by expressing the results as PFC per 10^6 cells cultured. It is clear that under optimal conditions (i.e. with 2-Me) the maximal response per 10^6 cells cultured is similar at both low and high cell densities. The data are consistent

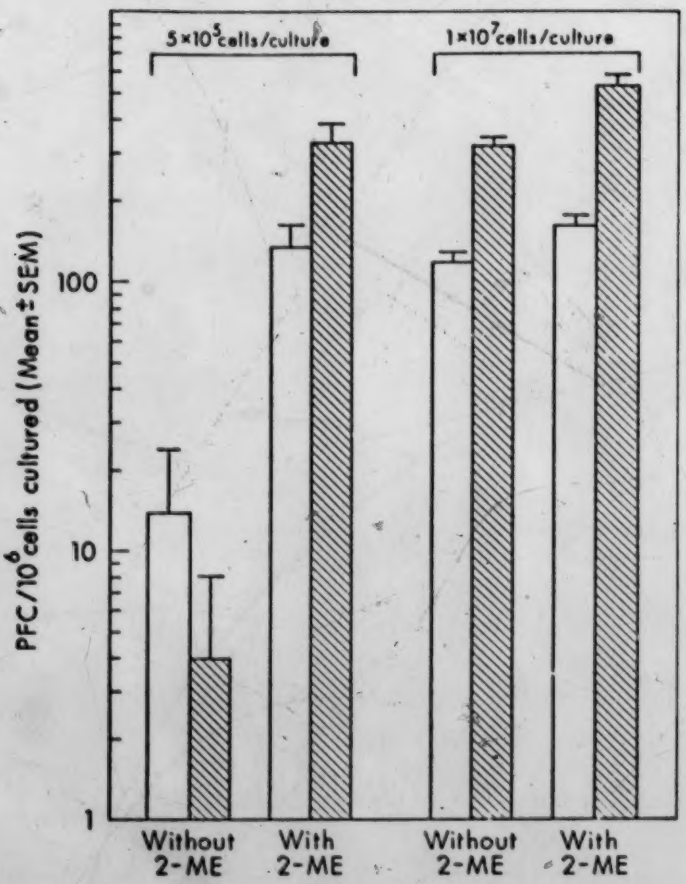


Fig. 4.1: The requirement for 2-Me at low cell density for an anti-SRBC response. CBA/J spleen cells were first incubated with 0.02% v/v SRBC $\pm$ 10^{-3} M dbcAMP in the absence of 2-Me. At 12 hr, the cells were washed three times with medium and recultured in quadruplicate in the Mishell-Dutton system at $5x10^5$ or $1x10^7$ cells per culture in the presence or absence of $5x10^{-5}$ M 2-Me. All cultures were assayed for AFC to SRBC at 108 hr. Unshaded columns, control cells; shaded columns, cells treated with 10^{-3} M dbcAMP for 12 hr first.

with the conclusion that a critical number of macrophages per culture is necessary, that dbcAMP may inhibit macrophages, and that its stimulatory effect is due to actions on other cell types.

In the presence of 2-Me the response of normal or dbcAMP treated cultures was linear with cell density in the 0.3 to $3x10^6$ cells per culture range, and tapered off at higher cell densities (Fig. 4.2).

The effect of dbcAMP on the AFC response to POL at different antigen concentrations

POL was shown to be a TI antigen in CBA/H mice (Feldmann and Basten, 1971). Also its concentration can be easily varied since it is a soluble antigen. These properties of POL make it a useful antigen to study B cell activation. If cAMP has a B cell effect, it might be expected to lower the antigen concentration needed for optimal antibody response to POL. The ability of dbcAMP to influence the anti-POL AFC response at different antigen concentrations was therefore investigated. CBA/J spleen cells were incubated with various concentrations of POL for the first 12 hr in the presence of dbcAMP. At 12 hr the cells were extensively washed to remove dbcAMP and unbound POL and recultured in the Diener-Armstrong system. The cultures were assayed at 108 hr. The results are shown in Fig. 4.3. Pretreatment with dbcAMP did not lead to lower antigen requirements; i.e. there is no shift in the dose-response curve. The stimulation by dbcAMP was highest at high antigen concentration (10^{-1} µg and 1 µg/ml) and only a small stimulatory effect was observed at low antigen concentrations (10^{-4} to 10^{-2} µg/ml). These results

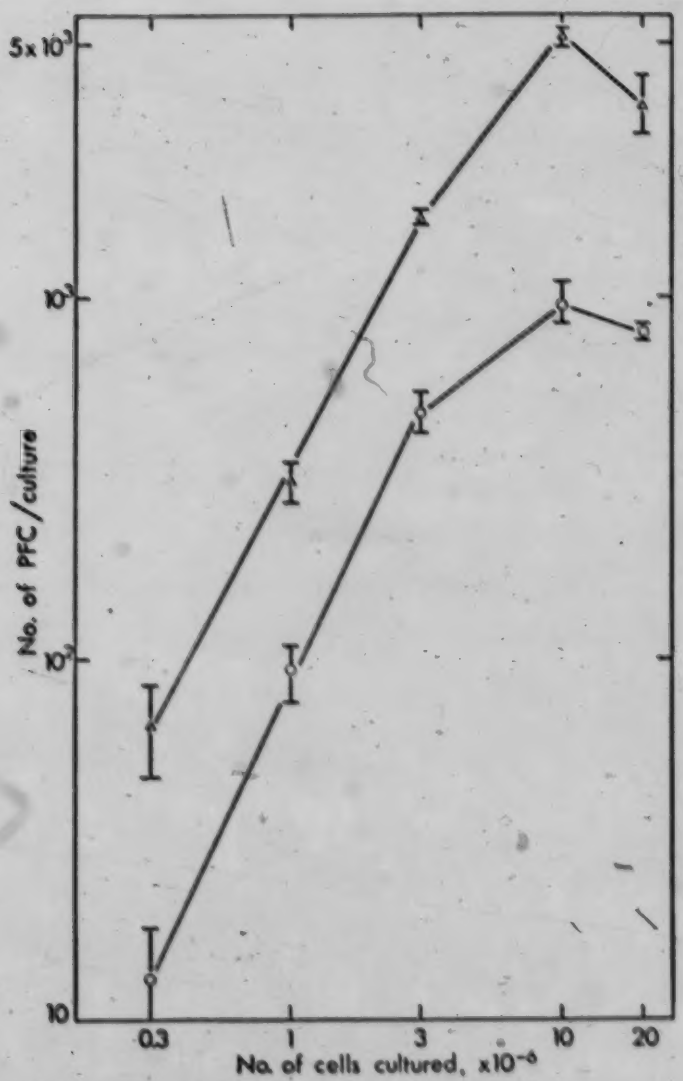


Fig. 4.2. The AFC response to SRBC at different cell densities. The spleen cells were pulsed with 10^{-3} M dbcAMP and 0.02% v/v SRBC as previously described. The cells were then recultured in the Mishell-Dutton system in quadruplicate at the indicated cell density (expressed as the number of viable cells at zero time). $5x10^{-5}$ M 2-Me was present throughout the 108 hr culture period. (O), control cells; (Δ), cells treated with 10^{-3} M dbcAMP for 12 hr first.

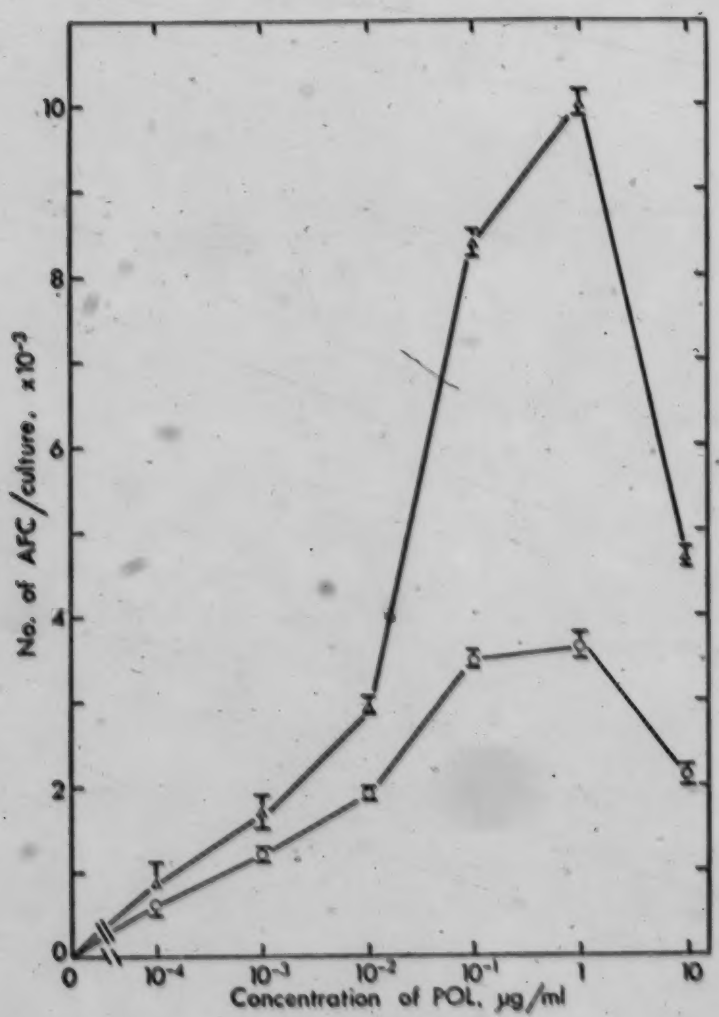


Fig. 4.3. The effect of dbcAMP on the AFC response to POL at different antigen concentrations. Spleen cells were incubated with various concentrations of POL with or without 10^{-3} M dbcAMP for 12 hr in Petri dishes. At 12 hr, the cells were extensively washed (four times in 10 ml of medium) to remove unbound POL and dbcAMP. The cells were then recultured in quadruplicate in the Diener-Armstrong system without any further addition of POL. The cultures were assayed for AFC to POL at 108 hr. (O), Cells + POL; (Δ), Cells + POL + 10^{-3} M dbcAMP.

suggest that cAMP has no direct B cell effect. However, as will be shown in later sections, cAMP can regulate the response to POL, a TI antigen, by exerting effects on T cells.

The effect of dbcAMP on ATXBM spleen cells

There have been reports to indicate that dbcAMP (Uzunova and Hanna, 1972) or poly A:U (Cone and Johnson, 1971) can partially replace T cells under in vivo conditions. In cultures depleted of T cells, dbcAMP can be examined for its ability to replace T cell function. Further, if dbcAMP has a B cell effect, it would be expected to stimulate the AFC response to POL, a TI antigen, under conditions of T cell depletion. The effects of dbcAMP on the AFC responses of CBA/J ATXBM (T cell-deprived) cultures to SRBC (a TD antigen) and POL were therefore investigated. The results are shown in Table 4.1. In non-dbcAMP treated cultures, the response of ATXBM spleen cells to SRBC was 29% of normal. This anti-SRBC response in ATXBM cultures is probably due to incomplete T cell depletion. With dbcAMP pretreatment, normal and ATXBM anti-SRBC responses were increased 2 fold and 3.4 fold respectively. Similar effects of dbcAMP were also observed for CBA/CaJ ATXBM cultures (data not shown). DbcAMP therefore appeared to replace T cell function since it enhanced the anti-SRBC response in ATXBM cultures to normal levels. However, it is not clear from this experiment if dbcAMP exerted its effects on ATXBM B cells through the residual T cell population (or T cell precursors), or affected the B cells directly.

Before dbcAMP pretreatment the anti-POL response of

Table 4.1

The effect of dbcAMP on the AFC responses of CBA/J ATXBM spleen cells to SRBC and POL

Additives at 0-12 hr	AFC/culture to SRBC (mean ± SEM)		AFC/culture to POL (mean ± SEM)	
	Normal	ATXBM	Normal	ATXBM
SRBC + POL	1535 ± 106	445 ± 38	1355 ± 110	865 ± 72
SRBC + POL + dbcAMP	3095 ± 161	1535 ± 117	3845 ± 343	1800 ± 101

Normal or ATXBM spleen cells were incubated with 0.02% v/v SRBC and 10 ng POL/ml ± 10^{-3} M dbcAMP for 12 hr in Petri dishes. The cells were washed three times in medium and recultured with antigen in the Mishell-Dutton culture system in quadruplicate. 2-Me was present throughout the experiment. All cultures were assayed at 108 hr.

ATXBM cultures was 64% of normal (Table 4.1). This suggested that the POL response in CBA/J is relatively independent of T cells. After dbcAMP treatment, the anti-POL response in normal and ATXBM cultures were enhanced by 2.8 fold and 2.1 fold respectively. This stimulation of the anti-POL response by dbcAMP was also observed in CBA/CaJ ATXBM cultures (data not shown). Since POL is a TI antigen, and cAMP could stimulate the response under conditions of T cell depletion the data suggest that cAMP affects B cells. But, as will be shown in the next experiment, cAMP has no direct B cell effect. Conclusions drawn from studies done in ATXBM cultures can be misleading because such cultures contain residual T cells or precursor T cells that can be readily activated (Scheid et al., 1973).

Mediation of cAMP actions by T cells

A more stringent procedure for T cell depletion involves the treatment of normal spleen cells with anti-θ serum and complement. CBA/J spleen cells were treated either with guinea pig complement alone (control) or with anti-θ serum and guinea pig complement as described in the Methods section. The effect of dbcAMP on the ability of control or anti-θ treated cells to respond to SRBC (a TD antigen) and POL (a TI antigen) was then examined. The results are shown in Table 4.2. It is clear from the data that dbcAMP can stimulate the AFC response to both SRBC and POL in cells treated with complement alone (2.1 fold and 2.6 fold, respectively). Cells treated with anti-θ serum plus complement lost their ability to respond to SRBC, as expected for a TD antigen.

Table 4.2

The effect of dbcAMP on the AFC responses of anti-θ treated CBA/J spleen cells to SRBC and POL

Additives at 0-12 hr	AFC/culture to SRBC (mean ± SEM) Normal[a]	Anti-θ[b]	AFC/culture to POL (mean ± SEM) Normal[a]	Anti-θ[b]
SRBC + POL	650 ± 72	43 ± 13	3000 ± 141	8830 ± 648
SRBC + POL + dbcAMP	1375 ± 190	68 ± 29	7890 ± 1112	8030 ± 621

[a]Cells treated with complement only.

[b]Cells treated with anti-θ serum and complement.

The concentrations of the materials used were: SRBC, 0.02% v/v; POL, 10 ng/ml; and dbcAMP, 10^{-3} M. Each culture contained the equivalent of $1x10^7$ viable spleen cells at 0 hr. $5x10^{-5}$ M 2-Me was present throughout the 108 hr culture period.

Futhermore, dbcAMP could not restore the anti-SRBC response. The response to the TI antigen, PGL, was stimulated by dbcAMP only with the control cells; T cell depletion led to a dbcAMP-insensitive state. Moreover, these T cell-depleted cultures gave as high a response as control cultures treated with dbcAMP. It is concluded that 1) dbcAMP cannot substitute for T cell function or cause the maturation of T cell precursors into functional T cells under these conditions, and 2) dbcAMP has no demonstrable, direct B cell effect in this system.

More surprisingly, anti-θ treatment alone enhanced the anti-PGL response of the treated cells by 2.9 fold. This enhancement was the same as occurred after treatment of control cells with dbcAMP. In other words, removal of θ-bearing (T) cells allows a greater response to a TI antigen, and increasing the intracellular cAMP level in antigen-sensitive T cells of normal spleen cultures does the same. The data which follow further support the hypothesis that the dbcAMP effect is exerted on a class of antigen-specific regulator (or "suppressor") cells.

Cell-mixing experiments

In Chapter III it was shown that the dbcAMP effect was apparently antigen-dependent and antigen-specific. A more rigorous test system would be to mix, at the end of 12 hr, cells which have had various treatments. In this system, all cells would be at a comparable stage of immune induction when mixed and thus able to benefit from any stimulatory events, which may be transient. Such experiments are described below. These data confirm the

earlier conclusion of antigen-specificity and antigen-dependence, but more importantly, clearly demonstrate that the action of dbcAMP is on a regulator, or suppressor cell.

Six groups of cells were set up and cultured during the 0 to 12 hr period as follows: 1) Cells + SRBC; 2) Cells + SRBC + dbcAMP; 3) Cells + POL; 4) Cells + POL + dbcAMP; 5) Cells alone; 6) Cells + dbcAMP. It was first necessary to establish that under these conditions the response was directly proportional to the cell number. That this was the case is shown in Table 4.3. Increasing the cell number from $3x10^6$ to $6x10^6$ cells per culture increased the response of normal and dbcAMP treated cultures to SRBC and POL by about two fold. At 12 hr the cells were mixed as indicated in Table 4.4. It is clear from the data that in the case of the AFC response to POL, the dbcAMP effect is both antigen-dependent and antigen-specific. In other words, the anti-POL response of cells treated with POL was enhanced only when cells that were treated with both POL and dbcAMP were added to it (line 1 versus line 2). The anti-POL response was not enhanced when cells treated either with SRBC or no antigen in the presence of dbcAMP were added to it (line 1 versus lines 4, 6).

However, the AFC response to SRBC shows a different pattern. The response of cells treated with SRBC only can be enhanced by pretreatment of cells in the following order: POL + dbcAMP > SRBC + dbcAMP > dbcAMP alone. Thus, the dbcAMP effect appears not to be antigen-specific nor antigen-dependent in this case. Since fetal calf serum in the medium has antigens which cross-react with SRBC (Mishell and Dutton, 1967), the

Table 4.3

The AFC response to SRBC or POL is linear with cell density in the $3x10^6$ to $6x10^6$ cells per culture range

No. of cells per culture	Additives at 0-12 hr	Anti-SRBC PFC/culture (mean ± SEM)	Anti-POL AFC/culture (mean ± SEM)
$3x10^6$	SRBC	610 ± 52	n.d.
$6x10^6$	SRBC	1260 ± 106	"
$3x10^6$	SRBC + dbcAMP	2270 ± 189	"
$6x10^6$	SRBC + dbcAMP	4515 ± 394	"
$3x10^6$	POL	n.d.	1062 ± 134
$6x10^6$	POL	"	2230 ± 243
$3x10^6$	POL + dbcAMP	"	2795 ± 120
$6x10^6$	POL + dbcAMP	"	4465 ± 305

The spleen cells were incubated with 0.02% v/v SRBC or 100 ng POL/ml ± 10^{-3} M dbcAMP for 12 hr in Petri dishes. After removal of dbcAMP, the cells were recultured with the appropriate antigen in the Mishell-Dutton system in quadruplicate. 2-Me was present throughout the 108 hr culture period.

Table 4.4

The effect of dbcAMP treated cells on the AFC response of non-dbcAMP treated cells

Cell groups mixed at 12 hr		Anti-POL AFC/culture (mean ± SEM)	Anti-SRBC PFC/culture (mean ± SEM)
$3x10^6$	$3x10^6$		
POL[a]	-	986 ± 104	n.d.
"	POL + dbcAMP[b]	3422 ± 83	"
"	SRBC[c]	1073 ± 54	"
"	SRBC + dbcAMP[d]	1160 ± 106	"
"	Cells[e]	1013 ± 455	"
"	Cells + dbcAMP[f]	1013 ± 104	"
SRBC[c]	-	n.d.	610 ± 52
"	SRBC + dbcAMP[d]	"	2025 ± 74
"	POL[a]	"	1180 ± 176
"	POL + dbcAMP[b]	"	2525 ± 116
"	Cells[e]	"	1185 ± 119
"	Cells + dbcAMP[f]	"	1585 ± 291

Spleen cells were incubated during the first 12 hr as follows:
[a]Cells + 100 ng POL/ml; [b]Cells + 100ng POL/ml + 10^{-3} M dbcAMP;
[c]Cells + 0.02% v/v SRBC; [d]Cells + 0.02% v/v SRBC + 10^{-3} M dbcAMP;
[e]Cells + medium; [f]Cells + medium + 10^{-3} M dbcAMP.
At the end of 12 hr the cell groups were washed, mixed, and, in the case of POL, the antigen was added. They were further cultured in quadruplicate in the Mishell-Dutton system. $5x10^{-5}$ M 2-Me was present throughout the 108 hr culture period.

carryover of the dbcAMP effect from cells treated with medium alone is perhaps not too surprising. The greater enhancing effect of cells that have been treated with POL and dbcAMP, furthermore, could be explained by the potential mitogenic properties of POL (Coutinho and Möller, 1973). In summary, then, antigen specificity and dependence under these conditions is seen for the POL response, but not the SRBC response. Under the experimental conditions used in Chapter III, both responses showed specificity for, and dependence on, antigen.

An unexpected result of the cell-mixing studies was that the enhanced response of cells treated with dbcAMP and antigen was suppressed by normal cells. The cells were mixed in the manner indicated in Table 4.5. The contribution by each partner (data not shown) was measured separately so that the responses expected on the basis of simple additivity, and those observed, could be compared. The results show that the elevated response of cells treated with both dbcAMP and POL can be suppressed by cells which have been incubated with SRBC ± dbcAMP, no antigen ± dbcAMP or POL alone. The enhanced response of cells treated with dbcAMP and SRBC can also be suppressed in a similar manner with the exception that cells treated with POL + dbcAMP not only did not suppress but gave an enhancement of the anti-SRBC response, as already noted. The data also rule out the possibility that dbcAMP acts solely by stimulating a T helper cell function since we would then not expect any suppression of the response by untreated cells. The general conclusion is that regulatory, or suppressor cells are normally present in spleen cell populations. The function of these cells can be inhibited by

Table 4.5

The inhibition of the dbcAMP stimulated AFC responses by cells

Cell groups mixed at 12 hr		Anti-POL AFC/culture	Anti-SRBC PFC/culture	% of
$3x10^6$	$3x10^6$	(mean ± SEM)	(mean ± SEM)	expected[a]
POL+dbcAMP	-	3233 ± 530[b]	n.d.	(100)
"	POL	3422 ± 225	"	81
"	SRBC	2100 ± 265	"	49
"	SRBC+dbcAMP	2233 ± 270	"	53
"	Cells	3046 ± 296	"	78
"	Cells+dbcAMP	2620 ± 338	"	64
SRBC+dbcAMP	-	n.d.	2270 ± 189[c]	(100)
"	SRBC	"	2025 ± 74	70
"	POL	"	1190 ± 102	45
"	POL+dbcAMP	"	3765 ± 560	131
"	Cells	"	1075 ± 21	39
"	Cells+dbcAMP	"	1935 ± 265	63

[a]Refers to the response expected if the unmixed groups treated identically (data not shown) contributed in simple additive fashion.
[b]The AFC response to $3x10^6$ cells treated with POL only was 986 ± 104.
[c]The PFC response to $3x10^6$ cells treated with SRBC only was 610 ± 52.
The conditions used for this experiment were similar to those described for Table 4.4.

pretreating the cells with antigen and dbcAMP for 12 hr. POL can also non-specifically inhibit the function of suppressor cells to SRBC in the presence of dbcAMP and fetal calf serum (cross-reactive antigen?).

It was predicted that if the regulator cell specific for SRBC carried the θ antigen, then the addition of anti-θ treated cells would not suppress the enhanced response of dbcAMP treated cultures. Furthermore, the radiosensitivity of the regulator cell could be determined by gamma-irradiating the cells. The effects of normal, anti-θ treated or gamma-irradiated (1500 rads in vitro) cells on the response of dbcAMP treated or normal cultures to SRBC were therefore investigated. The results are shown in Fig. 4.4. The two clear conclusions from this experiment are that treatment with anti-θ serum relieves a regulatory activity of normal cells, and that irradiated cells have essentially no inhibitory effect on the dbcAMP enhancement of the SRBC response. Thus, addition of $4x10^6$ complement-treated cells to $2x10^6$ dbcAMP-treated cells leads to a respoonse 56% as high as that seen when anti-θ plus complement treated cells were added. Irradiated cells, which are unable to proliferate and thereby add to the response (see the lower set of curves) do not inhibit the dbcAMP effect significantly.

The data in Fig. 4.4 at first appear to contradict in part those of Table 4.5. However, both experiments were reproducible. What is the basis for the apparently contradictory results: namely that in Table 4.5, addition of $3x10^6$ cells at 12 hr inhibited the response of dbcAMP plus SRBC-treated cultures, whereas in Fig 4.4, $4x10^6$ normal cells did not inhibit $2x10^6$ dbcAMP treated cells?

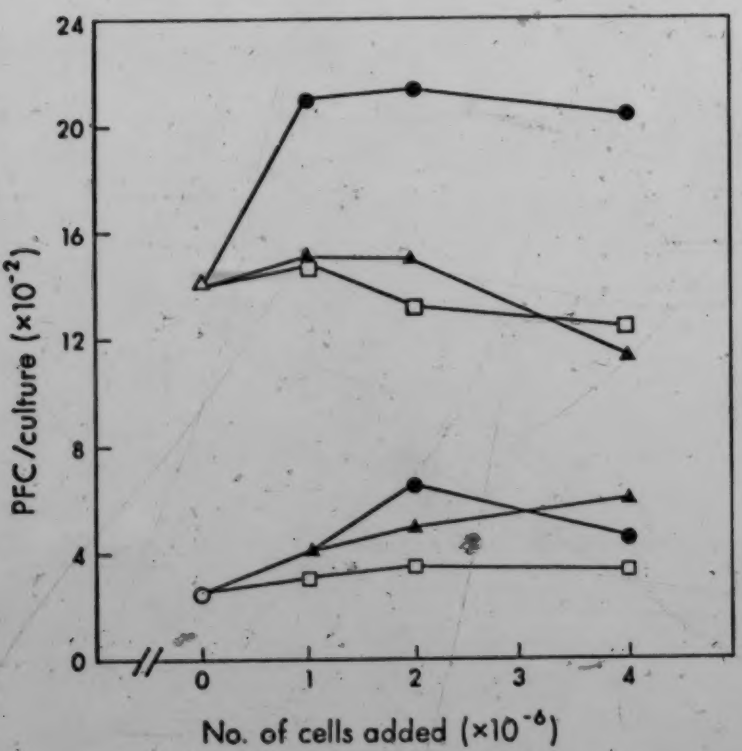


Fig. 4.4. Effect of normal, anti-θ treated or irradiated spleen cells on the anti-SRBC response of spleen cells stimulated by SRBC in the presence or absence of dbcAMP for 12 hr. Spleen cells were incubated with 0.02% v/v SRBC ± 10^{-3} M dbcAMP for 12 hr in Petri dishes. The cells were washed at 12 hr to remove dbcAMP and recultured with various numbers of complement treated, anti-θ treated or irradiated cells in triplicate in the Mishell-Dutton system. 2-Me was present throughout. All cultures were assayed at 108 hr. (○), $2x10^6$ cells + SRBC; (△), $2x10^6$ cells + SRBC + dbcAMP; (▲), complement treated cells; (●), anti-θ and complement treated cells; (□), irradiated cells. Anti-θ treated or irradiated cells stimulated with SRBC gave less than 50 PFC/10^7 cells whereas complement treated cells gave 650 PFC/10^7 cells.

Probably, that in the former case, the cells had been cultured for 12 hr with FCS present, and, as already stated, FCS contains cross-reactive antigens for SRBC. In Fig. 4.4, the untreated cells added at 12 hr were not "precultured". Thus, only in the experiments of Table 4.5 were the "normal" (suppressor containing) cells exposed to potential antigen. To explain the apparent contradiction, then, it is postulated that the antigen-specific suppressors are activated by antigen. Since the regulator (or suppressive) activity is radiation-sensitive, this is reasonable. It is also possible that complement may interfere with the regulatory activity.

The T-depleted cells in Fig. 4.4 appear to contribute to the response when added to dbcAMP-pretreated cells, but only to a limited degree. This may reflect the carryover of dbcAMP effect to subsequently added B cells, as expected if the dbcAMP effect is mediated by cells.

The enhancement of the AFC response to SRBC by SRBC-primed cells

The data described suggest that the magnitude of the immune response is regulated by the ratio of regulator to helper T cells. If normal cells have a greater number of regulator cells than those treated with dbcAMP, one would predict that the former would require a greater number of T helper cells to obtain a maximal AFC response. That this was the case is shown in Fig. 4.5. DbcAMP treated cultures require less T helper cells than normal cultures to achieve the maximum response. Futhermore, both dbcAMP treated and normal cultures reached the same maximum response at a sufficiently high T helper cell dose. This maximum value may reflect the conversion of all

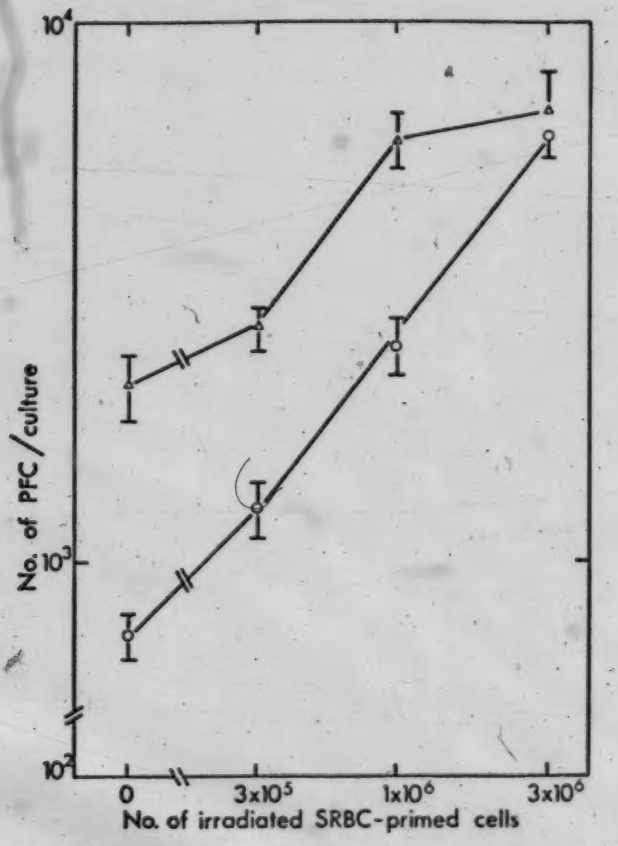


Fig. 4.5. The effect of irradiated-SRBC primed cells on the anti-SRBC response of normal or dbcAMP treated cells. The indicated number of irradiated SRBC-primed cells were added to normal or dbcAMP treated cells at 12 hr and allowed to remain until 108 hr, when all the cultures were assayed for AFC to SRBC. Each experimental group was done in quadruplicate in the Mishell-Dutton system in the presence of $5x10^{-5}$ M 2-Me. (O), $3x10^{6}$ cells + 0.02% v/v SRBC; (Δ), $3x10^{6}$ cells + 0.02% v/v SRBC + 10^{-3} M dbcAMP.

potential AFC to antibody synthesis, as will be discussed in Chapter V.

D. Discussion

The studies in this chapter show that dbcAMP can enhance the AFC response to either SRBC or POL through the inhibition of an antigen-stimulated, θ-bearing regulator (suppressor) cell. Irradiating normal spleen cells prior to antigenic stimulation abolished their suppressive activity. Dutton (1972) has reported the existence of short-lived, radiation-sensitive suppressor cells, which can be stimulated by Con A. Furthermore, irradiated spleen cells are a source of T helper cells when stimulated by Con A (Scavulli and Dutton, 1975). DbcAMP, histamine, prostaglandin E_1 and cholera endotoxin prevented the antigen-induced suppression of the antibody response to (T,G)-A--L (Mozes et al., 1974). The prevention of suppression in the present work, therefore, may be due to inhibition of antigen-stimulated regulator or suppressor cells by high intracellular levels of cAMP. This conclusion is strengthened by the observation that a splenic subpopulation bearing surface receptors for histamine can suppress humoral responses (Shearer et al., 1972). The AFC response to another TI antigen, SIII, is also regulated by T suppressor cells (Baker et al., 1974). In vivo treatment of mice with anti-lymphocyte or anti-thymocyte serum increased the AFC response to SIII by 15 to 20 fold. The ability of the anti-lymphocyte serum to induce enhancement was removed by absorption with thymocytes.

DbcAMP may inhibit the antigen-stimulated T regulator

cell either by 1) killing the cell, 2) suppressing its proliferation, or 3) preventing it from releasing soluble suppressive factors. The data do not allow us to distinguish between these possibilities. However, it is well known that cAMP can suppress T cell proliferative responses (Smith et al., 1971b; Greaves et al., 1974). Also there are many reports that cAMP can suppress the functions of effector cells. Examples are 1) the inhibition of the cytolytic activity of lymphocytes (Henney et al., 1972; Strom et al., 1972; Wolberg et al., 1975), 2) the inhibition of the formation of plaques by AFC (Melmon et al., 1974) and 3) the release of histamine from antigen-stimulated leukocytes (Bourne et al., 1974).

In Chapter III it was observed that cGMP, imidazole, or Con A could reverse the stimulation by dbcAMP (Table 3.6). In light of the present findings, it is possible that these compounds could act by reversing the inhibition of the T regulator cell by dbcAMP. The reversal of cAMP-mediated suppression by cGMP has been observed in lymphocytes (Watson et al., 1973) and other systems (Kram and Tomkins, 1973; Goldberg et al., 1975).

The greater stimulation of the anti-POL response in normal cultures at optimal antigen concentrations (Fig. 4.3) can also be explained on the basis of T regulator cells. At low antigen concentrations only high affinity cells are stimulated. The expression (i.e. antibody secretion) of these high affinity cells may be less strongly regulated by T cells. When a higher concentration is used, low affinity as well as high affinity cells for POL will be stimulated. The expression of the low affinity cells may be more susceptible to regulation by T cells. Hence, inhibition of the function

of these regulator cells by dbcAMP is expected to cause a relatively greater expression of the low affinity cells.

Enhancement of the anti-POL response following anti-θ treatment was not observed by Feldmann and Basten (1971). The data from ATXBM cells (Table 4.1) showed that the incomplete removal of T cells would not lead to an elevated POL response. However, since the ATXBM spleen cells used by Feldmann and Basten were obtained from animals drained of thoracic duct lymphocytes and anti-θ treated before use, it is unlikely that residual T cells could have accounted for the lack of stimulation of the POL response. Thus, a more likely explanation for the different results of their experiments and the present work is: 1) different culture systems were used (Marbrook versus Mishell-Dutton); and/or 2) the presence of 2-Me in the present experiments.

Poly A:U was reported to enhance the function of peritoneal exudate cells (Johnson and Johnson, 1971). On the other hand, the data presented here support the conclusion that dbcAMP inhibits the function of macrophages. The difference between these two observations could be because: 1) poly A:U does not act via cAMP and/or 2) splenic macrophages are more sensitive than peritoneal exudate cells to inhibition by cAMP.

CHAPTER V

THE REGULATION OF THE ANTIBODY RESPONSE BY T CELLS

A. Introduction

A characteristic feature of the immune response is the clonal proliferation of antigen-specific B cells into AFC. That cell replication is required for the induction and propagation of a primary response is well established (Dutton and Mishell, 1967; Rowley et al., 1968; Tannenberg and Malaviya, 1968; Szenberg and Cunningham, 1968; Perkins et al., 1969; Nakamura et al., 1972). Antigen-induced proliferation begins about 12 to 24 hr after antigenic stimulation (Dutton and Mishell, 1967; Perkins et al., 1969). PFC to SRBC can be detected as early as 24 hr after antigenic stimulation in vivo (Perkins et al., 1969). Single cell studies show that cells that are actively secreting antibody can undergo further cell division to produce two daughter AFC (Claflin and Smithies, 1967) and that the daughter AFC exhibit functional symmetry as judged by plaque morphology (Nossal and Lewis, 1971).

The doubling time for AFC in vivo during the exponential phase has been reported to be from 5 to 8 hr (Claflin and Smithies, 1967; Rowley et al., 1968; Tannenberg and Malaviya, 1968; Perkins et al., 1969). However, while all workers agree that AFC production requires proliferation, there is a disagreement as to whether the doubling time for AFC corresponds to the cell cycle

time. Using a double labelling technique Tannenberg and Malaviya (1968) found that the cell cycle time of AFC was considerably longer than the observed doubling time for PFC. The estimated cell cycle time was 13 hr (S phase = 8 hr; G1 = 3 hr; and G2 = 2 hr). A cell cycle time of about 14 hr was also found for lymphocytes stimulated by PHA or allogeneic cells by following the DNA synthetic activity of hydroxyurea-synchronized cultures (Lohrmann et al., 1974). On the other hand, Rowley et al. (1968) reported that the cell cycle time of AFC was determined by the strength of the antigenic stimulation. Thus, a high antigen level plus adjuvant could produce a cell cycle time as short as 5.6 hr whereas a weak antigen dose led to a value of 8.7 hr. These data were obtained from colchicine arrested cells and two of the assumptions used for the calculation of the cell cycle time were that 1) secretion of antibody by cells in interphase is not affected by colchicine, and 2) all AFC in mitosis, whether arrested in metaphase or not, do not form observable plaques. Since these assumptions remain to be proven, the weight of evidence still favours a cell cycle time of 13 to 14 hr for AFC.

A detailed kinetic analysis of the number of AFC in vivo showed that they increased in a "staircase" manner and not smoothly, as commonly thought (Perkins et al., 1969). These observations indicated that there was a considerable degree of synchronous growth during the course of the proliferation of progenitors of AFC or of their progeny. The number of AFC increased by an average of 3 fold and up to 6 fold per "staircase". The data suggested that the antigen-stimulated B cell population includes both secreting

and non-secreting; antigen-reactive cells, with both populations proliferating. The recruitment of non-secreting into secreting cells then adds to the increase in AFC. This recruitment, or conversion, accounts for a doubling time of AFC shorter than the actual cell cycle time. The concept of conversion of nonantibody-secreting to antibody-secreting cells has gained more direct experimental support from the work of Schimpl and Wecker (1972). They observed that AFC precursors to a TD antigen could proliferate following antigenic stimulation even in the absence of T cells. These cells were incapable of secreting antibody but they could be converted into antibody-secreting cells by the addition of a T cell factor at later times of the culture. These observations were confirmed by Dutton (1975).

In Chapter IV it was shown that dbcAMP increased the number of AFC to TD and TI antigens by inhibiting the function of a T regulator (suppressor) cell. Theoretically, the increase in the number of AFC in dbcAMP treated cultures could be due to 1) a more rapid proliferation of the AFC, 2) a greater degree of conversion of nonantibody-secreting cells to AFC, or 3) an increase in the number of cells responding initially to the antigen (i.e. increasing the number of "immunocompetent units").

B. Results

Kinetics of AFC formation

The increased number of AFC observed in dbcAMP treated cultures could arise from more rapid proliferation of the AFC precursors. Therefore, the number of AFC to SRBC was assayed at

daily intervals to determine the rate of appearance of AFC in normal or dbcAMP treated cultures. The results are shown in Fig. 5.1. In normal cultures the increase in the number of AFC was exponential in the 36 to 84 hr period. The number of AFC remained almost constant during the 84 to 132 hr period. On the other hand, dbcAMP (or AP) treated cultures had fewer AFC at early times but the number increased exponentially from 36 to 84 hr and continue to increase until 108 hr before levelling off. The average doubling time for AFC in normal cultures was 11 hr whereas in the dbcAMP (or AP) treated cultures it was 7 hr. There are two possible interpretations of the data. 1) The rate of appearance of AFC is proportional to their proliferation rate. If this were the case, then the rate of proliferation of AFC in dbcAMP (or AP) treated cultures is more rapid than in the controls. 2) There is a greater degree of conversion of nonantibody-secreting to secreting cells in treated cultures. In this case a difference in the proliferation rates between normal or treated cells need not be postulated. A direct method for measuring the cell cycle time of AFC in normal or treated cultures will be needed to distinguish between these two possibilities.

The smaller number of AFC in dbcAMP (or AP) treated cultures at early times is open to three interpretations: 1) there was a longer lag period for the proliferation of AFC in treated cultures; 2) less T cell helper factors for converting non-secreting to secreting cells were produced at early times in treated cultures; and 3) there were fewer responding cells in treated cultures at early times. These points will be considered in later sections.

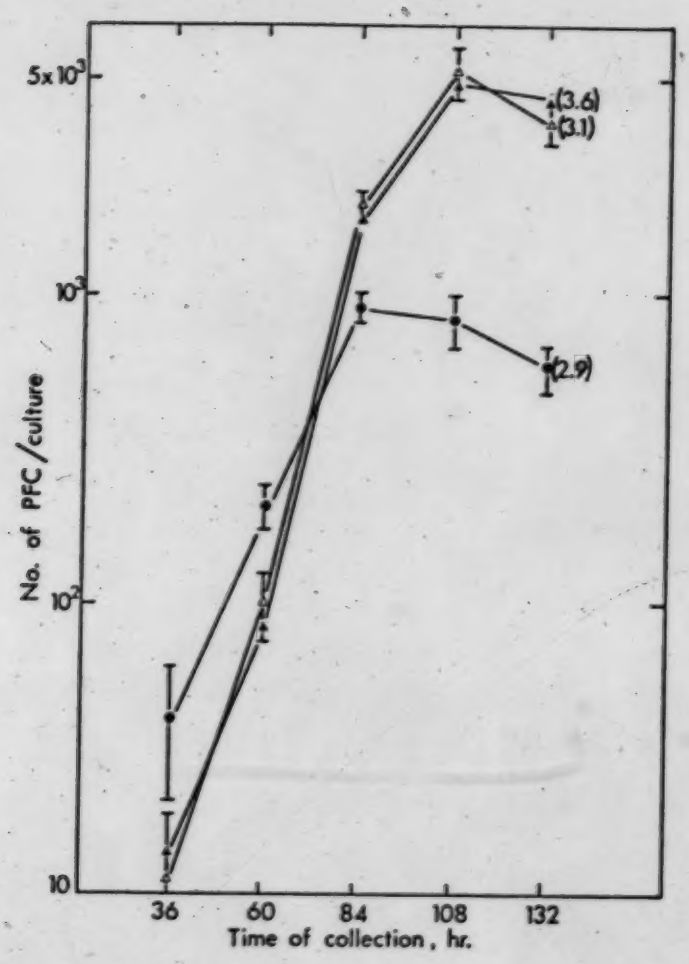


Fig. 5.1. Kinetics of the anti-SRBC response in cultures stimulated with dbcAMP or AP. Spleen cells were exposed to the agents indicated for the first 12 hr in Petri dishes. They were then washed and recultured in the Diener-Armstrong system in quadruplicate until the times indicated. Each culture was adjusted to contain 11×10^6 viable cells at the end of the 12 hr period. The numbers in parentheses indicate the number of viable cells, in millions, per culture at 132 hr. (O), untreated cells, given the same wash and incubation procedures; (Δ), cells treated with 10^{-3} M dbcAMP for 12 hr first; (▲), cells treated with 10^{-3} M AP for the same period. 0.01% v/v SRBC was present throughout the culture period, including the first 12 hr.

The kinetics of AFC responses to POL in normal or dbcAMP treated cultures (Fig. 5.2) are similar to those for SRBC. The doubling time for AFC was 11 hr for control, and 8 hr for dbcAMP treated, cultures.

Inhibition of AFC formation by cytosine-arabinoside

The dependence on proliferation for the formation of AFC to an antigen can be demonstrated by the use of cytosine-arabinoside (Ara-C). Ara-C is an effective but reversible inhibitor of DNA synthesis (Chu and Fischer, 1962). The minimum concentration at which Ara-C could completely suppress AFC formation to SRBC was $1x10^{-5}$ M (Table 5.1). At this concentration the overall viability of the cultured cells was not significantly affected, indicating that Ara-C had no general cytotoxic effects. To show that the formation of AFC to SRBC was dependent on proliferation, $1x10^{-5}$ M Ara-C were added to normal or dbcAMP treated cultures at the times indicated in Fig. 5.3 and allowed to remain in the cultures until the time of assay, i.e. 108 hr. The results show that the number of AFC obtained in normal or dbcAMP treated cultures was essentially frozen at the time of addition of Ara-C. The kinetics of the AFC response in Ara-C treated cultures was similar to that obtained by assaying the cultures at daily intervals (Fig. 5.1). The data are consistent with the interpretation that Ara-C stopped the proliferation of AFC. The AFC formed prior to the addition of inhibitor were still actively secreting antibody at the time of assay, i.e. 108 hr. The possibility remains that following the addition of Ara-C some conversion of nonantibody-secreting to antibody-

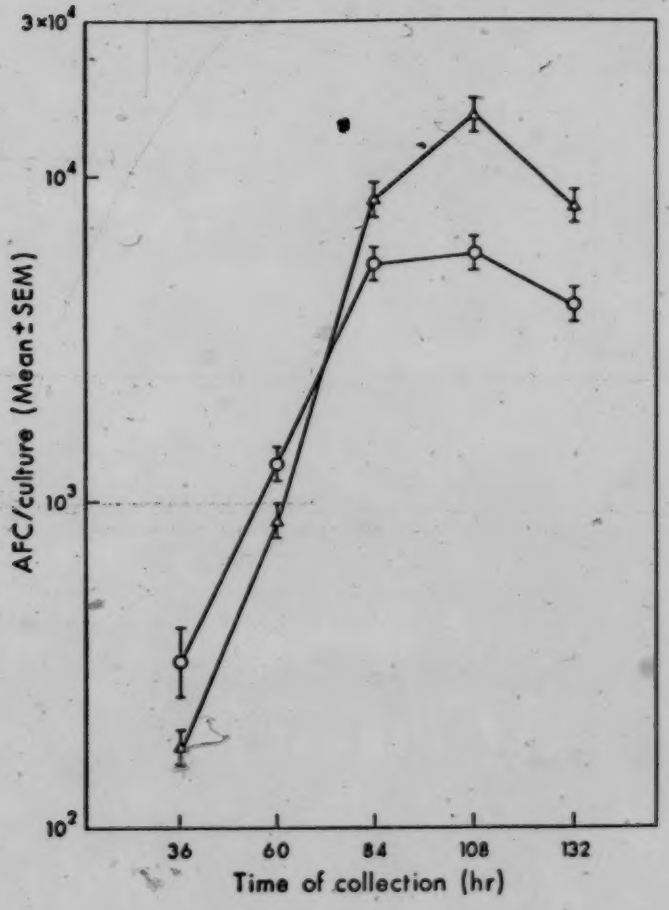


Fig. 5.2. Kinetics of the anti-POL response in cultures stimulated with dbcAMP. The conditions used were similar to those described for Fig. 5.1. with the exception that the antigen used was POL. Each flask contained the equivalent of 2×10^7 viable spleen cells at 0 hr. (O), cells + 100 ng POL/ml; (Δ), cells + 100 ng POL/ml + 10^{-3} M dbcAMP for 12 hr first.

Table 5.1

Inhibition of the AFC response to SRBC by cytosine-arabinoside

Concentration of Ara-C added	Anti-SRBC PFC/culture (mean ± SEM)	Anti-POL AFC/culture (mean ± SEM)	No. of cells recovered at 96 hr ($x10^{-6}$)
-	7341 ± 790	6240 ± 408	6.0
10^{-6} M	2791 ± 545	1793 ± 312	4.9
10^{-5} M	< 100	< 100	4.8

CBA/J spleen cells were cultured with 0.01% v/v SRBC and 250 ng POL/ml with the indicated concentration of Ara-C in the Diener-Armstrong system. Each group was performed in triplicate and each culture contained initially $2x10^7$ viable spleen cells. Ara-C was present throughout the experiment.

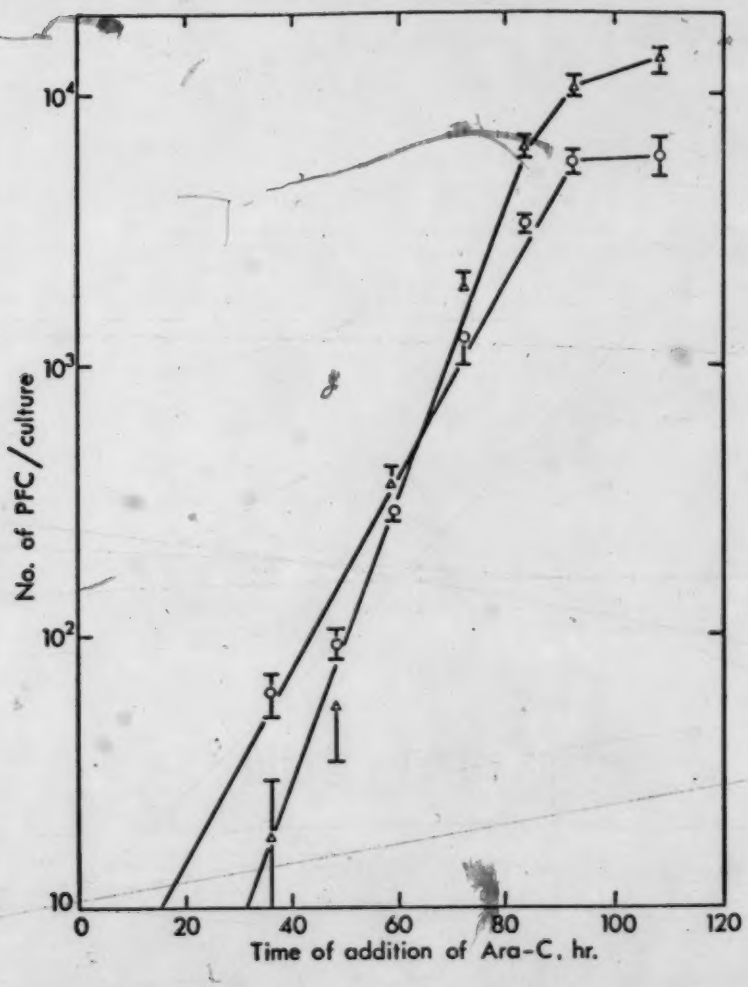


Fig. 5.3. The inhibition of the proliferation of anti-SRBC AFC by cytosine-arabinoside. Spleen cells were incubated with SRBC for 12 hr in Petri dishes in the presence or absence of dbcAMP. The cells were then washed and recultured with the equivalent number of 2×10^7 viable cells at 0 hr per flask in quadruplicate in the Diener-Armstrong system. Ara-C (1×10^{-5} M) was added to the cultures at the times indicated and left in the flasks until 108 hr, when all the cultures were assayed for AFC to SRBC. (○), cells + 0.01% v/v SRBC; (△), cells + 0.01% v/v SRBC + 10^{-3} M dbcAMP for 12 hr first.

secreting cells could occur, since this process may not be dependent on proliferation (Askonas et al., 1974).

The smaller number of AFC at early times in dbcAMP treated cultures could be due to a delay in proliferation of antigen-stimulated lymphocytes. The sensitivity of the cultures to Ara-C at early times was therefore investigated. The results are shown in Fig. 5.4. Both normal and dbcAMP treated cells were insensitive to inhibition by Ara-C during the 12 to 22 hr period, indicating that little or no proliferation was taking place during this period. However, during the 22 to 47 hr period, dbcAMP treated cells were more sensitive to inhibition by Ara-C than were normal cultures. The interpretation of these data is complicated by the greater sensitivity of dbcAMP treated cultures to washing at 35 hr. In fact, the dbcAMP effect disappears when the cells are washed at this time. The greater dependence of the SRBC response in dbcAMP treated cultures on a soluble factor is shown in Table 5.2. Washing the cells at 36 hr reduced the AFC response to SRBC in treated cultures to 44% of the response of the unwashed cultures. On adding back their own supernatants the responses of the washed cultures was partly reconstituted, to 61%. The normal response was not significantly decreased when cells were washed at 36 hr. By 60 hr, the AFC response of the dbcAMP treated cells was also relatively insensitive to washing and the readdition of its own supernatant did not improve the response. Thus, the critical period for the action of any soluble factor in dbcAMP treated cultures seems to be around 36 hr.

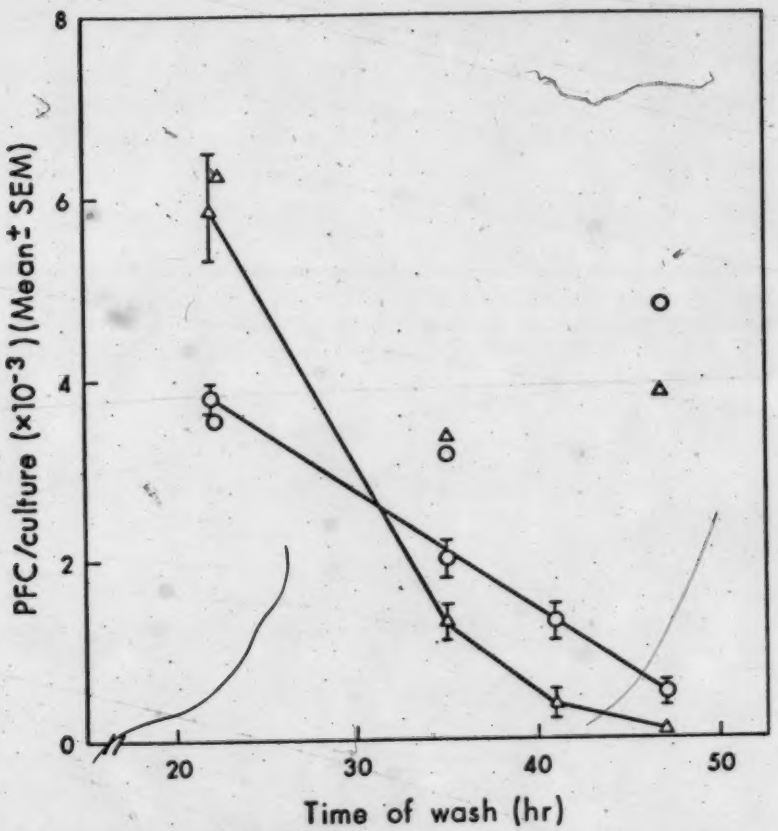


Fig. 5.4. Inhibition of the anti-SRBC response by cytosine-arabinoside at early times. Spleen cells were exposed to 10^{-3} M dbcAMP for the first 12 hr in Petri dishes in the presence of 0.01% v/v SRBC. The cells were then washed and recultured in quadruplicate with the equivalent number of $2x10^7$ viable cells at 0 hr per culture in the Diener-Armstrong system. $1x10^{-5}$ M Ara-C was added to half the cultures at 12 hr and removed by washing three times in medium at the times indicated. The cells were then recultured in culture flasks and assayed for AFC to SRBC at 108 hr. (O), untreated cells, given the same wash and incubation procedure; (O——O), untreated cells, given Ara-C at 12 hr; (Δ), dbcAMP treated cells, given the same wash and incubation procedures; (Δ——Δ), dbcAMP treated cells, given Ara-C at 12 hr.

Table 5.2

The dependence of dbcAMP treated cells on a soluble factor for AFC formation

Time of wash (hr)	AFC/culture to SRBC (mean ± SEM)			
	Normal		+ DbcAMP	
	+ Supernatant	+ Fresh medium	+ Supernatant	+ Fresh medium
12	-	6233 ± 580	-	20233 ± 1300
12; 36	6166 ± 1060	4700 ± 808	12333 ± 366	9000 ± 986
	(0.3 < P < 0.4)		(P = 0.05)	
12; 60	8200 ± 722	8900 ± 1136	15666 ± 768	15233 ± 1308

Spleen cells were incubated with 0.01% v/v SRBC ± 10^{-3} M dbcAMP for 12 hr. At 12 hr the cells were washed three times with medium and recultured in the Diener-Armstrong system at $3x10^7$ cells per culture. At 36 or 60 hr duplicate of four normal or dbcAMP treated cultures were harvested and their supernatants collected. The cells were washed once more with medium and resuspended in either 4 ml of own supernatant or 4 ml of fresh medium. The cells were then recultured in quadruplicate in the Diener-Armstrong system. All cultures were assayed at 108 hr.

The numbers of immunocompetent units

One mechanism by which dbcAMP can increase the number of AFC to SRBC is to increase the number of immunocompetent units (IU), defined as the minimum combination of interacting cells which will give a positive AFC response to a specific antigen. This hypothesis can be tested directly by determining the frequencies of IU in normal or dbcAMP treated cultures. The probability of nonresponse is derived from Poisson statistics and described by the equation $P(0) = e^{-\phi x}$ (Halsall and Makinodan, 1974), where P(0) is the probability of nonresponse (zero IU) in a culture containing x cells, and ϕ is the frequency of IU in the cell population. Thus, at limiting cell numbers, a plot of log P(0) versus the cell number should yield a straight line. This is shown in Fig. 5.5. The number of spleen cells containing one SRBC IU was calculated to be 1.3×10^5 for normal and 0.96×10^5 for dbcAMP treated cultures. This small difference in the frequency of responding units is insufficient to account for the 3 to 5 fold increase in the number of AFC normally observed for dbcAMP treated cultures. It also rules out the possibility that the smaller number of AFC seen at early times for dbcAMP treated cultures was due to a smaller number of responding units in these cultures.

The number of AFC obtained per IU, or clone size, was calculated using the equation AFC per IU =

$$\frac{\text{Total number of plaques obtained X number of cells containing one IU}}{\text{Total number of cells cultured}}$$

It was noted that the clone size of both normal and dbcAMP treated cultures increased with increasing cell number (Fig. 5.5). This

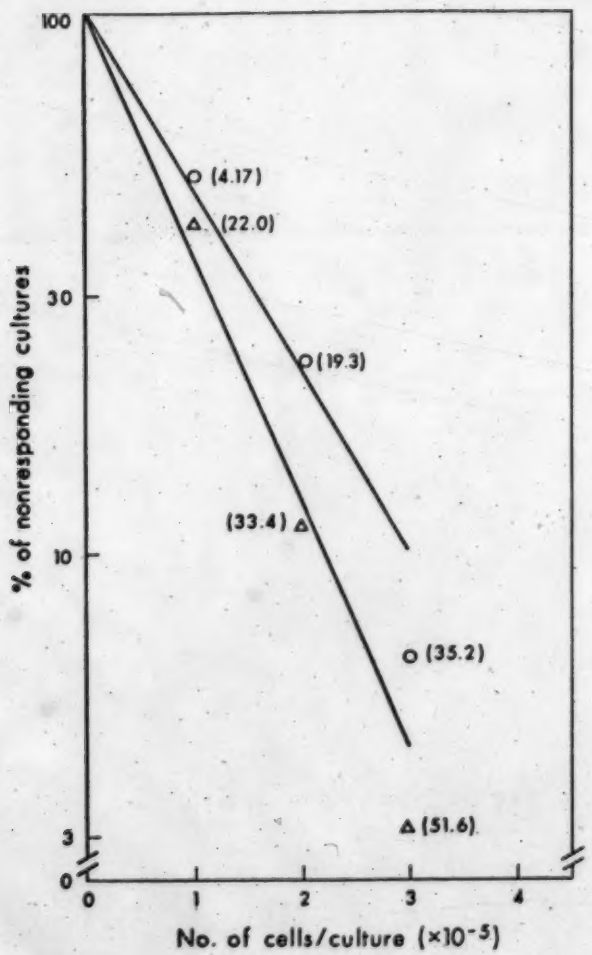


Fig. 5.5. The frequency of SRBC immunocompetent units in normal and dbcAMP treated cells. Spleen cells were exposed to 10^{-3} M dbcAMP for 12 hr in Petri dishes in the presence of 0.02% v/v SRBC. The cells were then washed and recultured with 0.02% v/v SRBC at the indicated cell density (expressed as the number of viable cells at 12 hr) in the Mishell-Dutton system. The cultures were assayed individually for AFC to SRBC at 108 hr. Each point represents results from 32 cultures. Cultures containing more than 4 PFC were scored as responding to SRBC. The numbers inparentheses represent the clone sizes at the indicated cell densities. (O), untreated cells; (Δ) cells treated with 10^{-3} M dbcAMP for 12 hr first.

suggests that T helper cells, and perhaps other factors, were limiting under these conditions.

The distribution of the number of AFC per culture in all the limiting dilution assays is shown in Fig. 5.6. The results show that the effect of dbcAMP treatment was the shift of cultures with small clone size to larger clone size.

Since the conversion of nonantibody-secreting to antibody-secreting cells is mediated by T helper factors (see Introduction) it is conceivable that T suppressor cells inhibit directly or indirectly the action of T helper factors. In other words, normal or dbcAMP treated cultures may proliferate to the same extent but dbcAMP treated cultures have more AFC because of a greater degree of conversion. The maximal AFC capacities of dbcAMP treated and normal cultures to SRBC should also be fully expressed when an excess of T helper factors is added. The results of adding $2x10^6$ irradiated SRBC-primed cells to cultures containing either $1x10^5$ normal or dbcAMP treated cells are shown in Fig. 5.7. Both normal and dbcAMP treated cultures gave the same average number of AFC per culture under these conditions indicating that the potential AFC forming capacities in both groups were indeed the same. Similar conclusions were obtained when excess T helper cells were added to $3x10^6$ normal or dbcAMP treated cells (Fig. 4.5). Essentially all the cultures in both groups gave a positive response, confirming the conclusion that at $1x10^5$ cells per culture, T cells, and not B cells, were limiting. The number of normal cultures giving small clone sizes was also greatly reduced (compare Figs. 5.6 and 5.7) with a proportional increase in the number of cultures with large

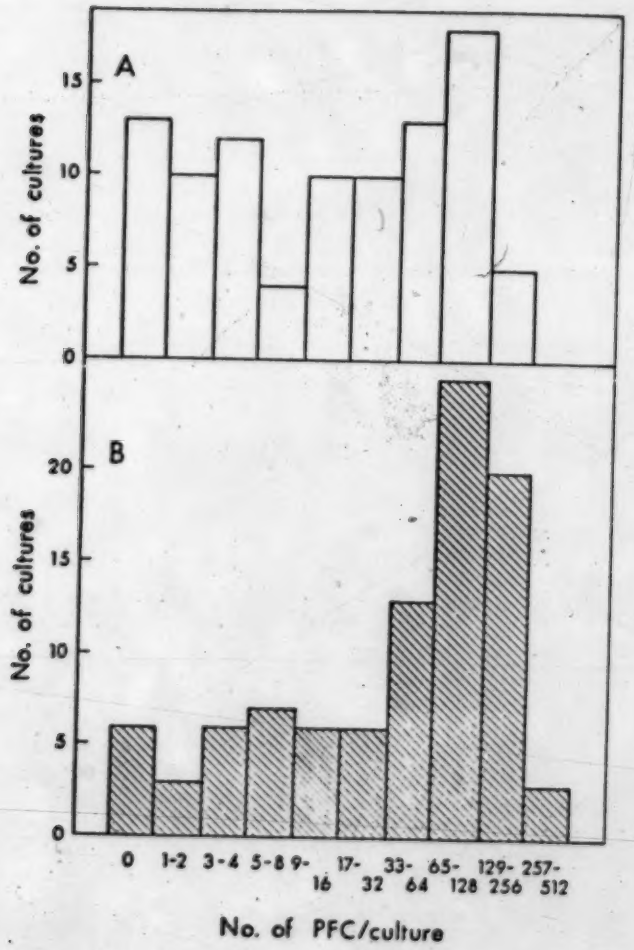


Fig. 5.6. Distribution of the number of PFC per culture in limiting cell dilution assays. The data for this figure were obtained from Fig. 5.5. The number of cultures containing different number of PFC are shown on the ordinate. The number under each column show the range of PFC scored in that column. (A), untreated cells; (B), cells treated with 10^{-3} M dbcAMP for 12 hr first.

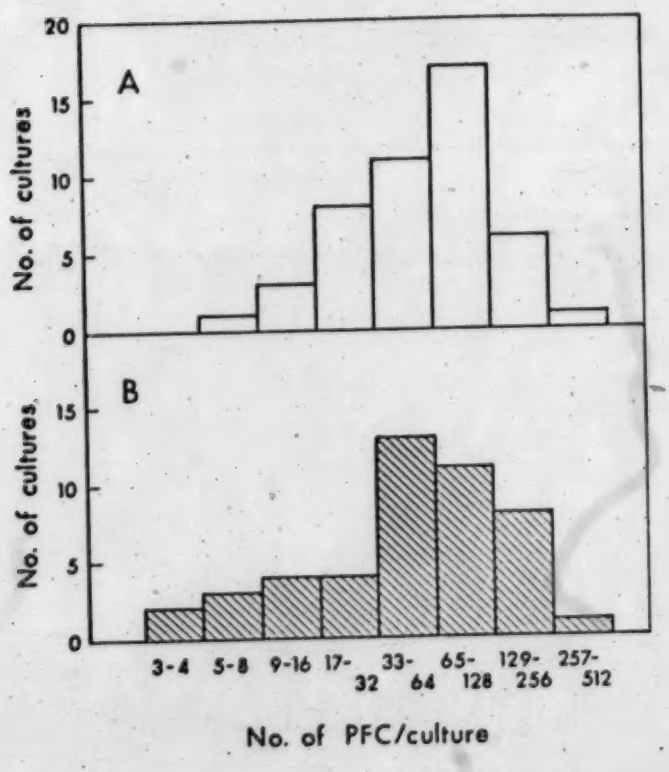


Fig. 5.7. The effect of irradiated SRBC-primed cells on the distribution of the number of PFC per culture under limiting cell dose conditions. Spleen cells were exposed to 10^{-3} M dbcAMP for the first 12 hr in Petri dishes in the presence of 0.02% v/v SRBC. The cells were then washed and recultured at $1x10^5$ viable cells per culture. $2x10^6$ irradiated SRBC-primed cells and 0.02% v/v SRBC were added to each culture. The cells were assayed individually for AFC to SRBC at 108 hr. (A), untreated cells; (B), cells treated with 10^{-3} M dbcAMP for 12 hr first. The average number of PFC was 75 per culture for untreated cells and 79 per culture for treated cells. The total number of cultures was 47 for A and 46 for B.

clones.

In the presence of excess T helper cells, the number of normal cells containing one IU to SRBC was calculated to be 3.0×10^4 (Fig. 5.8). Thus, the frequency of IU in normal cultures can be increased by 4 fold in the presence of excess T help.

C. Discussion

The studies in this chapter show that the increase in the number of AFC in dbcAMP treated cultures is due to 1) a shorter doubling time for AFC, and 2) a longer period of AFC formation. The frequency of immunocompetent units in dbcAMP treated and normal cultures differs by only about 30% and is insufficient to account for the 3 to 5 fold increase in AFC normally observed in dbcAMP treated cultures. The cell cycle time in dbcAMP treated cultures may be the same as normal since the elevated AFC response can arise from a greater degree of conversion from nonantibody-secreting to antibody-secreting cells in treated cultures.

It is important to note that at 40 hr there were about 20 AFC in dbcAMP treated cultures (11×10^6 viable cells at 12 hr, Fig. 5.1), and the addition of Ara-C at this time yielded about the same number of AFC at 108 hr (ca 20 AFC per 14×10^6 viable cells, Fig. 5.3). Therefore, it seems unlikely that a significant number of nonsecreting, antigen-reactive cells are being converted to AFC in the absence of proliferation.

The sensitivity of dbcAMP treated cultures to washing was maximal at 35 hr (Fig. 5.4). However, reconstitution of the washed cultures by its own supernatant was incomplete (44% to 61%, Table 5.2).

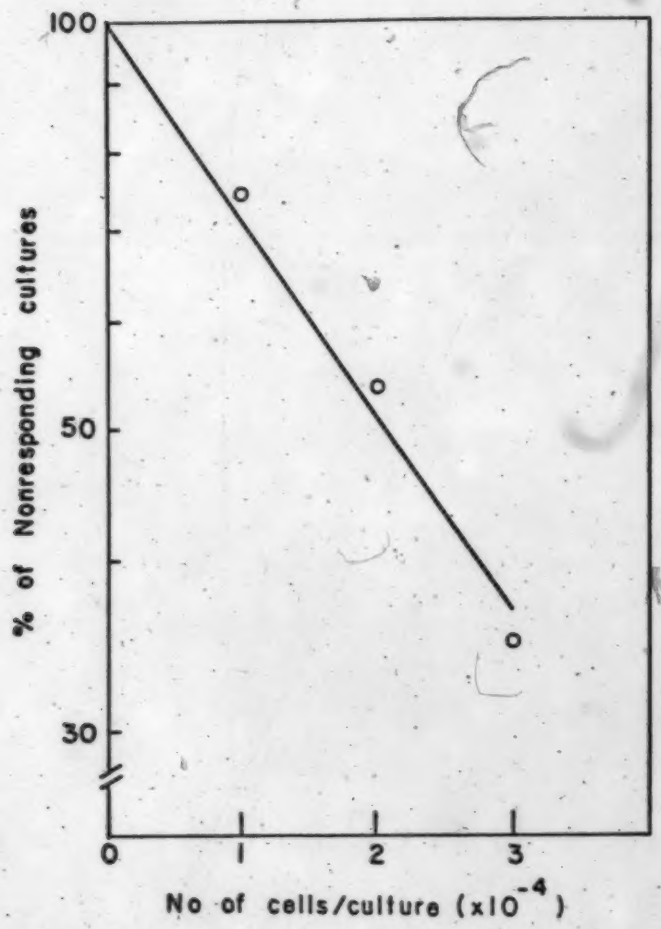


Fig. 5.8. Effect of irradiated SRBC-primed cells on the frequency of SRBC immunocompetent units in normal spleen cells. Normal spleen cells were incubated with 0.02% v/v SRBC for 12 hr. At the end of 12 hr the cells were washed and recultured with $2x10^6$ irradiated SRBC-primed spleen cells and 0.02% v/v SRBC at the indicated cell density. The cultures were assayed individually for AFC to SRBC at 108 hr. Each point represents the results from 64 cultures. Cultures containing more than 4 PFC were scored as responding to SRBC.

This could be due to the "local concentration" of the soluble factor on the readdition of its own supernatant being less that that of unwashed cultures. Schimpl and Wecker (1972, 1975) also reported that the soluble T cell factor required for converting proliferating cells to AFC had maximal effects when added at 24 hr or 48 hr after B cell stimulation by a TD antigen (SRBC).

DbcAMP treatment led to more cultures with larger clone size under limiting cell dilution conditions (Fig. 5.6). Since dbcAMP treated cells had fewer functional T regulator cells (see Chapter IV) under these conditions dbcAMP treated cultures would have fewer functional T regulator cells seeded to them. Consequently, more of the treated cultures would have a T helper to T regulator ratio higher than that of normal cultures, i.e. a bigger clone size. Normal cultures can be made to give the same distribution profile of AFC per culture as dbcAMP treated cultures by adding excess T helper cells (Fig. 5.7). Thus, the inhibitory effects of T regulator cells on the formation of AFC can be overcome by excess T help. Under conditions of maximal conversion the difference in clone sizes between individual cultures of the same group is likely due to differences in the affinities of antigen-specific B cells for antigen. B cells with high affinity for antigen are expected to go through more number of cell divisions whereas low affinity B cells have a very limited capacity for cell division following stimulation by antigen. In other words, cultures with large clones are those that contained high affinity cells.

Before proposing a model for the regulation of AFC formation by T cells a summary of the relevant findings is necessary. It is

well established that the formation of AFC to a TD antigen requires the collaboration between T and B cells (Katz and Benacerraf, 1972). Because of such a collaborative process much of the discussion on mechanisms of T-B cell collaboration has centered on 2 signals (i.e. antigen and T cells) being required for the initiation of B cell proliferation. However, it was reported recently that proliferation of B cells to a TD antigen could occur following their interaction with antigen alone (Hunig et al., 1974; Dutton, 1975). T cell factors were required only for the conversion of the non-antibody-secreting B cells to AFC (Schimpl and Wecker, 1972; Dutton, 1975). In other words, the 2 signals required for the formation of AFC to a TD antigen could act in a sequential manner. Detailed kinetic studies also showed that the rate of formation of AFC to SRBC in vivo in normal mice could be accounted for only if there were conversion of nonantibody-secreting to antibody-secreting cells (Perkins et al., 1969). It is likely that once the conversion has taken place, the progeny of the AFC will all be antibody-secreting cells because single cell studies showed that the daughter cells of AFC exhibited functional symmetry (Nossal and Lewis, 1971). These findings are summarized in Fig. 5.9.

Studies with dbcAMP showed that the AFC responses to SRBC and POL are controlled by T regulator cells. The T regulator cell probably does not affect the initiation of antibody responses since its inhibition did not result in more cells being triggered (Fig. 5.5) or in a lowering of the antigenic dose required for optimum stimulation (Fig. 4.3). The increased number of AFC in dbcAMP treated cultures must therefore be due either to a greater degree of

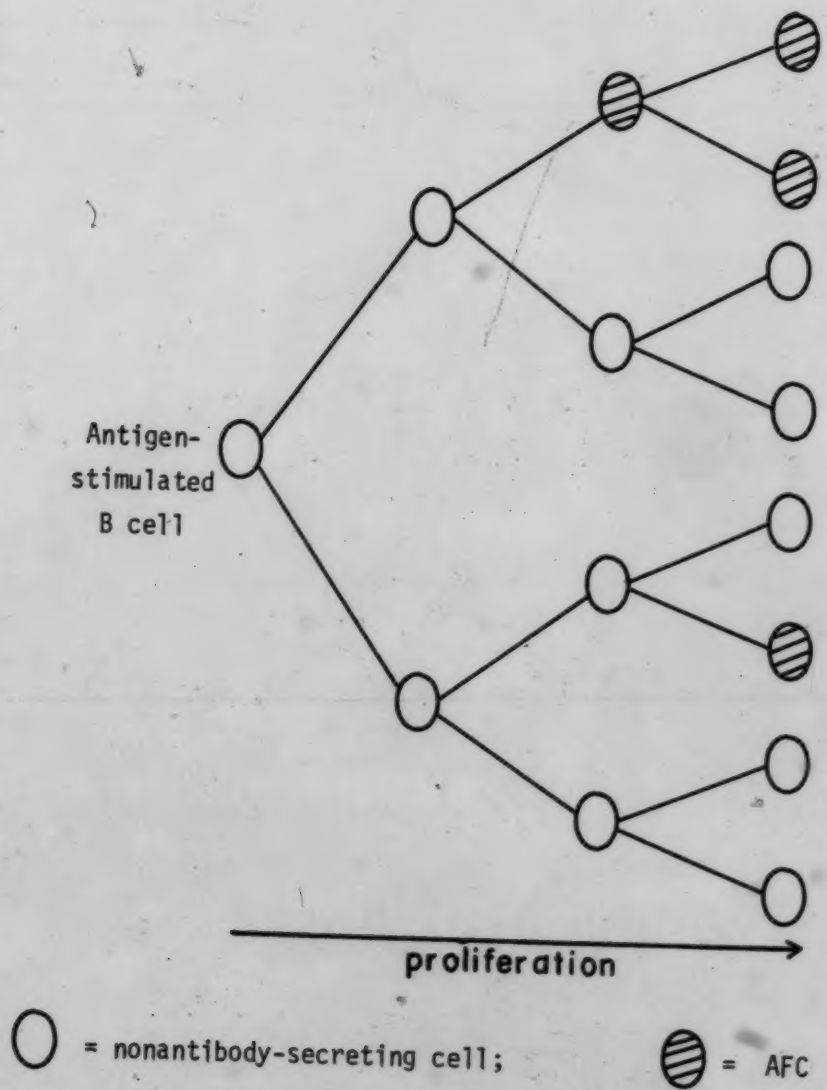


Fig. 5.9. A simplified scheme for the formation of AFC. Antigen-reactive B cells, when stimulated by a TD antigen, can proliferate in the absence of T cells to give rise to nonantibody-secreting cells. The conversion of nonantibody-secreting cells to AFC is mediated by T cell helper factors.

conversion of nonantibody-secreting to antibody-secreting cells or to a more rapid proliferation rate. Lohrmann et al. (1974) have shown that the cell cycle time of lymphocytes is independent of the strength of the antigenic stimulus; lymphocytes stimulated with allogeneic cells or different concentrations of PHA all showed a mean cell cycle time of 14 $\pm$ 0.6 hr. By labelling Con A-stimulated thymocytes first with ^{3}H-thymidine and then with bromodeoxyuridine, Gerhart and Paetkau (personal communication) were able to show that the average time required for the DNA of proliferating lymphocytes to shift from light-light to light-heavy was 12 to 13 hr. Thus, although there is no direct evidence, it is reasonable to postulate that the AFC in normal and dbcAMP treated cultures have the same cell cycle time of about 13 hr. The alternative view that the function of the T regulator cell is to control conversion of non-antibody-secreting cells to AFC is therefore favoured. In the case of the AFC response to SRBC, T regulator cells are postulated to inhibit the conversion of nonantibody-secreting to antibody-secreting cells by T cell helper factors. This regulation can be overcome by adding excess T help. Thus, assuming a cell cycle time of 13 hr, an exponential phase of 24 to 108 hr (Figs. 5.1, 5.2, and 5.3), and the number of normal spleen cells containing one IU to be 3.0×10^{4} (Fig. 5.8), the expected number of AFC/10^{6} cells cultured is 2900 at 108 hr, if complete conversion has occurred. The observed value in the presence of optimum T help was 2300 AFC/10^{6} cells (Fig. 4.5), which is 79% of the predicted value.

The AFC response to the TI antigen, POL, is also postulated to be regulated by T regulator cells since removal of these cells

by anti-θ treatment results in the formation of more AFC (Table 4.2). However, AFC responses to TI antigens differ from responses to TD antigens in that B cells stimulated by TI antigens can become AFC without T helper factors.

The advantage of regulator cells is that they provide a mechanism by which a pool of nonantibody-secreting cells can be preserved. This pool of cells may be the source of memory cells. Thus, conversion to PFC, and then to plasma cells, effectively removes a clone from further responses. Cells which remain antigen-reactive but not converted may provide the B memory component for secondary responses. Thus, regulation of conversion to AFC may insure immunological memory. Implicit in this model is the assumption that the proliferation of antigen-reactive cells is self-regulatory. In other words, the number of cell divisions these cells can go through is determined by the strength of the antigenic stimulus.

A direct test of this model is the determination of the cell cycle times of AFC in normal and dbcAMP treated cultures. The hypothesis will be strengthened if the cell cycle times turn out to be the same. On the other hand, if the cell cycle time of AFC in dbcAMP treated cultures is significantly shorter than in normal cultures, the hypothesis will be disproved.

BIBLIOGRAPHY

ADA, G.L., BYRT, P. 1969. Specific inactivation of antigen-reactive cells with ^{125}I-labelled antigen. Nature 222: 1291-1292.

ADA, G.L., NOSSAL, G.J.V., PYE, J., ABBOTT, A. 1964. Antigen in immunity. I. Preparation and properties of flagellar antigens from Salmonella adelaide. Aust. J. Exp. Biol. Med. Sci. 42: 267-282.

ANDERSSON, J., MELCHERS, F. 1973. Induction of immunoglobulin M synthesis and secretion in bone marrow-derived lymphocytes by locally concentrated Concanavalin A. Proc. Nat. Acad. Sci. 70: 416-420.

AOKI, T., HAMMERLING, U., HARVEN, E., BOYSE, E.A., OLD, L.J. 1969. Antigenic structure of cell surfaces. An immunoferritin study of the occurrence and topography of H-2, θ and TL alloantigens on mouse cells. J. Exp. Med. 130: 979-1001.

ASKONAS, B.A., SCHIMPL, A., WECKER, E. 1974. The differentiation function of T cell replacing factor in nu/nu spleen cell cultures. Eur. J. Immunol. 4: 164-169.

BACH,F., HIRSCHHORN, K. 1963. γ-globulin production by human lymphocytes in vitro. Exp. Cell. Res. 32: 592-595.

BAKER, P.J., PRESCOTT, B., STASHAK, P.W., AMSBAUGH, D.F. 1974. Regulation of the antibody response by thymic-derived cells. In The Immune System (Sercarz, E.E., Williamson, A.R., Fox, C.F., eds.), p. 415-429.

BARNETT, R.E., SCOTT, R.E., FURCHT, L.T., KERSEY, J.H. 1974. Evidence that mitogenic lectins induce changes in lymphocyte membrane fluidity. Nature 249: 465-466.

BEAVO, J.A., HARDMAN, J.G., SUTHERLAND, E.W. 1970. Hydrolysis of cyclic guanosine and adenosine 3',5'-monophosphates by rat and bovine tissues. J. Biol. Chem. 245: 5649-5655.

BOSING-SCHNEIDER, R., KOLB, H. 1973. Influence of cyclic AMP on early events of the immune induction. Nature 244: 224-225.

BOURNE, H.R., LICHTENSTEIN, L.M., MELMON, K.L., HENNEY, C.S., WEINSTEIN, Y., SHEARER, G.M. 1974. Modulation of inflammation and immunity by cyclic AMP. Science 184: 18-28.

BRAUN, W., ISHIZUKA, M. 1971a. Antibody formation: Reduced responses after administration of excessive amounts of nonspecific stimulators. Proc. Nat. Acad. Sci. 68: 1114-1116.

BRAUN, W., ISHIZUKA, M. 1971b. Cyclic AMP and immune responses. II. Phosphodiesterase inhibitors as potentiators of polynucleotide effects on antibody formation. J. Immunol. 107: 1036-1042.

BREINL, R., HAUROWITZ, F. 1930. Z. Phys. Chem. 192: 45.

BRETSCHER, P. 1972. The control of humoral and associative antibody synthesis. Transplant. Rev. 11: 217-267.

BRETSCHER, P., COHN, M. 1968. Minimal model for the mechansim of antibody induction and paralysis by antigen. Nature 220: 444-448.

BRUNNER, K.T., MAUEL, J., CEROTTINI, J.C., CHAPUIS, B. 1968. Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. Immunol. 14: 181-196.

BURGER, M.M., BOMBIK, B.M., BRECKENRIDGE, B. McL., SHEPPARD, J.R. 1972. Growth control and cyclic alterations of cyclic AMP in the cell cycle. Nature New Biol. 239: 161-163.

BURNET, F.M. 1959. The clonal selection theory of acquired immunity. Nashville, Vanderbilt University Press, 208 p.

CANTOR, H., ASOFSKY, R. 1972. Synergy among lymphoid cells mediating the graft-versus-host response. III. Evidence for interaction between two types of thymus-derived cells. J. Exp. Med. 135: 764-779.

CEROTTINI, J.C., NORDIN, A.A., BRUNNER, K.T. 1970. Specific in vitro cytotoxicity of thymus-derived lymphocytes sensitized to alloantigens. Nature 228: 1308-1309.

CHEN, C., HIRSCH, J.G. 1972. The effects of mercaptoethanol and of peritoneal macrophages on the antibody-forming capacity of nonadherent mouse spleen cells in vitro. J. Exp. Med. 136: 604-617.

CHU, M.Y., FISCHER, G.A. 1962. A proposed mechanism of action of 1-β-D-arabinofuranosyl-cytosine as an inhibitor of the growth of leukemic cells. Biochem. Pharmacol. 11: 423-430.

CLAFLIN, A.J., SMITHIES, O. 1967. Antibody-producing cells in division. Science 157: 1561-1562.

CLAMAN, H.N., CHAPERON, E.A. 1969. Immunologic complementation between thymus and marrow cells. A model for the two-cell theory of immunocompetence. Transplant. Rev. 1: 92-113.

CLAMAN, H.N., CHAPERON, E.A., TRIPLETT, R.F. 1966. Thymus-marrow cell combinations. Synergism in antibody production. Proc. Soc. Exp. Biol. Med. 122: 1167-1171.

CLICK, R.E., BENCK, L., LATER, B.J. 1972. Enhancement of antibody synthesis in vitro by mercaptoethanol. Cell Immunol. 3: 156-160.

COFFINO, P., GRAY, J., TOMKINS, G.M. 1975. Dibutyryl cAMP synchronizes S49 mouse lymphoma cells in G1. In Advances in Cyclic Nucleotide Research, Vol. 5 (Drummond, G.I., Greengard, P., Robison, G.A., eds.), p. 831.

COHEN, A., SCHLESINGER, M. 1970. Absorption of guinea pig serum with agar. A method for elimination of its cytoxicity for murine thymus cells. Transplant. 10: 130-132.

COHN, M., BLOMBERG, B., GECKELER, W., RASCHKE, W., RIBLET, R., WEIGERT, M. 1974. First order considerations in analyzing the generator of diversity. In The Immune System (Sercarz, E.E., Williamson, A.R., Fox, C.F., eds.), p. 89-117.

CONE, R.E., JOHNSON, A.G. 1971. Regulation of the immune system by synthetic nucleotides. III. Action on antigen-reactive cells of thymic origin. J. Exp. Med. 133: 665-676.

COOPER, M.D., PETERSON, R.D.A., SOUTH, M.A., GOOD, R.A. 1966. The functions of the thymus system and the bursa system in the chicken. J. Exp. Med. 123: 75-102.

COUTINHO, A., MOLLER, G. 1973. B cell mitogenic properties of thymus-independent antigens. Nature New Biol. 245: 12-14.

COUTINHO, A., MOLLER, G. 1974. Immune activation of B cells: evidence for 'one non-specific triggering signal' not delivered by the Ig receptors. Scand. J. Immunol. 13: 133-146.

CROSS, M.E., ORD, M.G. 1971. Changes in histone phosphorylation and associated early metabolic events in pig lymphocyte cultures transformed by phytohemagglutinin or 6-N,2'-O-dibutyryl-adenosine 3',5'-cyclic monophosphate. Biochem. J. 124: 241-248.

CUNNINGHAM, A., SZENBERG, A. 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunol. 14: 599-600.

DIENER, E. 1968. A new method for the enumeration of single antibody-producing cells. J. Immunol. 100: 1062-1070.

DIENER, E., ARMSTRONG, D.W. 1969. Immunological tolerance in vitro: Kinetic studies at the cellular level. J. Exp. Med. 129: 591-603.

DREYER, W.J., BENNETT, J.C. 1965. The molecular basis of antibody formation: A paradox. Proc. Nat. Acad. Sci. 54: 864-869.

DUTTON, R.W. 1972. Inhibitory and stimulatory effects of concanavalin A on the response of mouse spleen cell suspensions to antigen. I. Characterization of the inhibitory cell activity. J. Exp. Med. 136: 1445-1460.

DUTTON, R.W. 1973. Inhibitory and stimulatory effects of concanavalin A on the response of mouse spleen cell suspensions to antigen. II. Evidence for separate stimulatory and inhibitory cells. J. Exp. Med. 138: 1496-1505.

DUTTON, R.W. 1975. Separate signals for the initiation of proliferation and differentiation in the B cell response to antigen. Transplant. Rev. 23: 66-77.

DUTTON, R.W., MISHELL, R.I. 1967. Cell populations and cell proliferation in the in vitro response of normal mouse spleen to heterologous erythrocytes. Analysis by the hot pulse technique. J. Exp. Med. 126: 443-454.

EDELMAN, G.M., GALLY, J.A. 1967. Somatic recombination of duplicated genes: An hypothesis on the origin of antibody diversity. Proc. Nat. Acad. Sci. 57: 353-358.

EHRLICH, P. 1900. On immunity with special reference to cell life. Proc. Roy. Soc., Series B, 66: 424-448.

EPSTEIN, C.J., GOLDBERGER, R.F., ANFINSEN, C.B. 1963. The genetic control of tertiary protein structure: Studies with model systems. Cold Spring Harbour Symp. Quant. Biol. 28: 439-449.

FELDMANN, M., BASTEN, A. 1971. The relationship between antigenic structure and the requirements for thymus-derived cells in the immune response. J. Exp. Med. 134: 103-119.

FELDMANN, M., GREAVES, M.F., PARKER, D.C., RITTENBERG, M.B. 1974. Direct triggering of B lymphocytes by insolubilized antigen. Eur. J. Immunol. 4: 591-597.

FELDMANN, M., NOSSAL, G.J.V. 1972. Tolerance, enhancement and the regulation of interactions between T cells, B cells and macrophage. Transplant. Rev. 13: 3-34.

FISHER, D.B., MUELLER, G.C. 1971. Studies on the mechanism by which phytohemagglutinin rapidly stimulates phospholipid metabolism of human lymphocytes. Biochim. Biophys. Acta 248: 434-448.

GERSHON, R.K. 1974. T cell control of antibody production. In Contemporary Topics in Immunobiology, Vol. 3 (Cooper, M.D., Warner, N.L., eds), p. 1.

GOLDBERG, N.D., HADDOX, M.K., NICOL, S.E., GLASS, D.B., SANFORD, C.H., KUEHL, Jr., F.A., ESTENSEN, R. 1975. Biologic regulation through opposing influences of cyclic GMP and cyclic AMP: The Yin Yang hypothesis. In Advances in Cyclic Nucleotide Research, Vol. 5 (Drummond, G.I., Greengard, P., Robison, G.A., eds.), p. 307-230.

GOOD, R.A., BIGGARS, W.D., PARK, B.H. 1971. In Progress in Immunology (Amos, B. ed.), p. 699.

GREAVES, M.F., BAUMINGER, S. 1972. Activation of T and B lymphocytes by insoluble mitogens. Nature New Biol. 235: 67-70.

GREAVES, M., JANOSSY, G. 1972. Elicitation of selective T and B lymphocyte responses by cell surface binding ligands. Transplant. Rev. 11: 87-130.

GREAVES, M., JANOSSY, G., FELDMANN, M., DOENHOFF, M. 1974. Polyclonal mitogens and the nature of B lymphocyte activation mechanisms. In The Immune System (Sercarz, E.E., Williamson, A.R., Fox, C.F., eds.), p. 271-297.

HADDEN, J.W., HADDEN, E.M., HADDOX, M.K., GOLDBERG, N.D. 1972. Guanosine 3',5'-cyclic monophosphate. A possible intracellular mediator of mitogenic influences in lymphocytes. Proc. Nat. Acad. Sci. 69: 3024-3027.

HALSALL, M.K., MAKINODAN, T. 1974. Analysis of the limiting-dilution assay used for estimating frequencies of immunocompetent units. Cell. Immunol. 11: 456-465.

HANKS, J.H., WALLACE, J.H. 1958. Determination of cell viability. Proc. Soc. Exp. Biol. Med. 98: 188-192.

HEERSCHE, J.N.M., FEDAK, S.A., AURBACH, G.D. 1971. The mode of action of dibutyryl adenosine 3',5'-monophosphate on bone tissue in vitro. J. Biol. Chem. 246: 6770-6775.

HENNEY, C.S., BOURNE, H.R., LICHTENSTEIN, L.M. 1972. The role of cyclic AMP in the specific cytolytic activity of lymphocytes. J. Immunol. 108: 1526-1534.

HOOD, L., PRAHL, J. 1971. The immune system: a model for differentiation in higher organisms. Adv. in Immunol. 14: 291-351.

HOOD, L., TALMAGE, D.W. 1970. Mechanism of antibody diversity: Germ line basis for variability. Science 168: 325-334.

HUMPHREY, J.H., KELLER, H.U. 1970. Some evidence for specific interaction between immunologically competent cells and antigens. In Developmental aspects of antibody formation and structure (Sterzl, J., Riha, I., eds), p. 485-502.

HUNIG, T., SCHIMPL, A., WECKER, E. 1974. Autoradiographic studies on the proliferation of antibody-producing cells in vitro. J. Exp. Med. 139: 754-760.

HUNTER, P., MUNRO, A.J. 1972. Response of mice to rabbit Fab'_2 and Fab'. Thymus independence of IgM response and thymus dependence of IgG response. Immunol. 23: 69-81.

ISHIZUKA, M., BRAUN, W., MATSUMOTO, T. 1971. Cyclic AMP and immune responses. I. Influence of poly A:U and cyclic AMP on antibody formation in vitro. J. Immunol. 107: 1027-1035.

ISHIZUKA, M., GAFIN, M., BRAUN, W. 1970. Cyclic AMP effects on antibody formation and their similarities to hormone-mediated events. Proc. Soc. Exp. Biol. Med. 134: 963-967.

JAROSLOW, B.N., ORTIZ-ORTIZ, L. 1972. Influence of PolyA:Poly U on early events in the immune response in vitro. Cell. Immunol. 3: 123-132.

JERNE, N.K. 1955. The natural selection theory of antibody formation. Proc. Nat. Acad. Sci. 41: 849-857.

JOHNSON, H.G., JOHNSON, A.G. 1971. Regulation of the immune system by synthetic polynucleotides. II. Action on peritoneal exudate cells. J. Exp. Med. 133: 649-664.

JOHNSON, G.S., PASTAN, I. 1972. $N^6,O^{2'}$-dibutyryl adenosine 3',5'-monophosphate induces pigment production in melanoma cells. Nature New Biol. 237: 267-268.

KATZ, D.H., BENACERRAF, B. 1972. The regulatory influence of activated T cells on B cell responses to antigen. Adv. Immunol. 15: 1-94.

KETTMAN, J., DUTTON, R.W. 1971. Radioresistance of the enhancing effect of cells from carrier-immunized mice in an in vitro primary immune response. Proc. Nat. Acad. Sci. 68: 699-703.

KILLANDER, D., RIGLER, R. 1969. Activation of deoxyribonucleoprotein in human leukocytes stimulated by phytohemagglutinin. I. Kinetics of the binding of acridine orange to deoxyribonucleoprotein. Exp. Cell Res. 54: 163-170.

KLEINSMITH, L.J., ALLFREY, V.G., MIRSKY, A.E. 1966. Phosphorylation of nuclear protein early in the course of gene activation in lymphocytes. Science 154: 780-781.

KOMURO, K., BOYSE, E.A. 1973. Induction of T lymphocytes from precursor cells in vitro by a product of the thymus. J. Exp. Med. 138: 479-482.

KRAMS, R., TOMKINS, G.M. 1973. Pleiotypic control by cAMP: interaction with cGMP and possible role of microtubules. Proc. Nat. Acad. Sci. 70: 1659-1663.

KRISHNARAJ, R., TALWAR, G.P. 1973. Role of cyclic AMP in mitogen induced transformation of human peripheral leukocytes. J. Immunol. 111: 1010-1017.

LANDSTEINER, K. 1947. The Specificity of Serological Reactions, rev. ed. Harvard Univ. Press, Cambridge, Massachusetts, 310p.

LEDERBERG, J. 1959. Genes and antibodies. Science 129: 1649-1653.

LEE, K.C., SHIOZAWA, C., SHAW, A., DIENER, E. 1975. Requirement for accessory cells in the antibody response to T cell dependent antigens in vitro. In preparation.

LOEB, L.A., AGARWAL, S.S., WOODSIDE, A.M. 1968. Induction of DNA polymerase in human lymphocytes by PHA. Proc. Nat. Acad. Sci. 61: 827-834.

LOHRMANN, H.P., GRAW, C.M., GRAW, Jr., R.G. 1974. Stimulated lymphocyte cultures. Responder recruitment and cell cycle kinetics. J. Exp. Med. 139: 1037-1048.

MACDONALD, H.R., CEROTTINI, J.C., BRUNNER, K.T. 1974. Generation of cytotoxic T lymphocytes in vitro. III. Velocity sedimentation studies of the differentiation and fate of effector cells in long-term mixed leukocyte cultures. J. Exp. Med. 140: 1511-1521.

MACMANUS, J.P., WHITFIELD, J.F., YOUDALE, T. 1971a. Stimulation by epinephrine of adenyl cyclase activity, cyclic AMP formation, DNA synthesis and cell proliferation in populations of rat thymic lymphocytes. J. Cell Physiol. 77: 103-116.

MACMANUS, J.P., WHITFIELD, J.F., BRACELAND, B. 1971b. Biochem. Biophys. Res. Commun. 42: 503-509.

MAINO, V.C., GREEN, N.M., CRUMPTON, M.J. 1974. The role of calcium ions in initiating transformation of lymphocytes. Nature 251: 324-327.

MANNING, D.D., REED, N.D., SHAFFER, C.F. 1973. Maintenance of skin xenografts of widely divergent phylogenetic origin on congenitally athymic (nude) mice. J. Exp. Med. 138: 488-494.

MARCHALONIS, J.J. 1974. Molecular and functional properties of lymphocyte surface immunoglobulin. In The Immune System (Sercarz, E.E., Williamson, A.R., Fox, C.F., eds), p.141-173.

MELMON, K.L., BOURNE, H.R., WEINSTEIN, Y., SHEARER, G.M., KRAM, J., BAUMINGER, S. 1974. Hemolytic plaque formation by leukocytes in vitro. Control by vasoactive hormones. J. Clin. Invest. 53: 13-21.

MILLER, J.F.A.P. 1960. Studies on mouse leukemia. The role of the thymus in leukemogenesis by cell-free leukemic filtrates. Brit. J. Cancer 14: 93-98.

MILLER, J.F.A.P. 1961. Immunological function of the thymus. Lancet 2: 748-749.

MILLER, J.F.A.P., MITCHELL, G.F. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. J. Exp. Med. 128: 801-820.

MILLER, J.F.A.P., MITCHELL, G.F. 1969. Thymus and antigen-reactive cells. Transplant. Rev. 1: 3-42.

MILLER, R.G., PHILLIPS, R.A. 1975. Development of B lymphocytes. Fed. Proc. 34: 145-150.

MISHELL, R.I., DUTTON, R.W. 1967. Immunization of dissociated spleen cell cultures from normal mice. J. Exp. Med. 126: 423-442.

MITCHISON, N.A. 1971a. The carrier effect in the secondary response to hapten-protein conjugates. I. Measurement of the effect with transferred cells and objections to the local environment hypothesis. Eur. J. Immunol. 1: 10-17.

MITCHISON, N.A. 1971b. Cell cooperation in the immune response: the hypothesis of an antigen presentation mechanism. Immunopath. 6: 52.

MOORE, M.A.S., METCALF, D. 1970. Ontogeny of the hemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. Brit. J. Haematol. 18: 279-296.

MOORE, M.A.S., OWEN, J.J.T. 1965. Chromosome marker studies on the development of the hemopoietic system in the chicken embryo. Nature 208: 989-990.

MOORE, M.A.S., OWEN, J.J.T. 1967. Stem cell migration in developing myeloid and lymphoid systems. Lancet 2: 658-659.

MOSIER, D.E. 1967. A requirement for two cell types for antibody formation in vitro. Science 158: 1573-1575.

MOZES, E., WEINSTEIN, Y., BOURNE, H.R., MELMON, K.L., SHEARER, G.M. 1974. In vitro correction of antigen-induced immune suppression: Effects of histamine, dbcAMP, and cholera enterotoxin. Cell. Immunol. 11: 57-63.

MUNRO, A.J., TAUSSIG, M.J. 1975. Two genes in the major histocompatibility complex control immune response. Nature 256: 103-106.

NAKAMURA, I., SEGAL, S., GLOBERSON, A., FELDMAN, M. 1972. DNA replication as a prerequisite for the induction of primary antibody response. Cell. Immunol. 4: 351-366.

NAOR, D., SULITZEANU, D. 1967. Binding of radioiodinated bovine serum albumin to mouse spleen cells. Nature 214: 687-688.

NOSSAL, G.J.V., ADA, G.L. 1971. Antigens, lymphoid cells and the immune response. Academic Press, New York/London, p.66-68.

NOSSAL, G.J.V., CUNNINGHAM, A., MITCHELL, G.F., MILLER, J.F.A.P. 1968. Cell to cell interaction in the immune response. III. Chromosomal marker analysis of single antibody-forming cells in reconstituted, irradiated, or thymectomized mice. J. Exp. Med. 128: 839-853.

NOSSAL, G.J.V., LEWIS, H. 1971. Functional symmetry amongst daughter cells arising in vitro from single antibody-forming cell. Immunol. 20: 739-753.

NOVOGRODSKY, A., KATCAHLSKI, E. 1970. Effect of PHA and prostaglandins on cyclic AMP synthesis in rat lymph node lymphocytes. Biochim. Biophys. Acta 215: 291-296.

PAULING, L. 1940. A theory of the structure and process of formation of antibodies. J. Amer. Chem. Soc. 62: 2643-2657.

PERKINS, E.H., SADO, T., MAKINODAN, T. 1969. Recruitment and proliferation of immunocompetent cells during the log phase of the primary antibody response. J. Immunol. 103: 668-678.

PETERS, J.H., HAUSEN, P. 1971. Effect of PHA on lymphocyte membrane transport. Eur. J. Biochem. 19: 502-508.

PLESCIA, O.J., YAMAMOTO, I., SHIMAMURA, T. 1975. Cyclic AMP and immune responses: changes in the splenic level of cyclic AMP during the response of mice to antigen. Proc. Nat. Acad. Sci. 72: 888-891.

POGO, B.G.T. 1969. The effect of N-acetyl-D-galactosamine and amantadine hydrochloride on equine lymphocytes stimulated with PHA. J. Cell Biol. 40: 571-575.

POGO, B.G.T., ALLFREY, V.G., MIRSKY, A.E. 1966. RNA synthesis and histone acetylation during the course of gene activation in lymphocytes. Proc. Nat. Acad. Sci. 55: 805-812.

QUASTEL, M.R., DOW, D.S., KAPLAN, J.G. 1970. Stimulation of ^{42}K-uptake into lymphocytes by PHA and the role of intracellular K^+ in lymphocyte transformation. In Proceedings 5th Leukocyte Culture Conference. Academic press, New York, p.97.

RAFF, M.C. 1969. Theta isoantigen as a marker of thymus-derived lymphocytes in mice. Nature 224: 378-379.

RAFF, M.C. 1970. Role of thymus derived lymphocytes in the secondary humoral response in mice. Nature 226: 1257-1258.

REIF, A.E., ALLEN, J.M.V. 1964. The AKR thymic antigen and its distribution in leukemias and nervous tissues. J. Exp. Med. 120: 413-433.

ROBISON, G.A., BUTCHER, R.W., SUTHERLAND, E.W. 1968. Cyclic AMP. Ann. Rev. Biochem. 37: 149-174.

ROSEMAN, J. 1969. X-ray resistant cell required for the induction of in vitro antibody formation. Science 165: 1125-1127.

ROWLEY, D.A., FITCH, F.W., MOSIER, D.E., SOLLIDAY, S., COPPLESON, L.W., BROWN, B.W. 1968. The rate of division of antibody-forming cells during the early primary immune response. J. Exp. Med. 127: 983-1002.

RUBIN, A.D., COOPER, H.L. 1965. Evolving patterns of RNA metabolism during transition from resting state to active growth in lymphocytes stimulated by PHA. Proc. Nat. Acad. Sci. 54: 469-476.

RUDLAND, P.S., SEELEY, M., SEIFERT, W. 1974. Cyclic GMP and cyclic AMP levels in normal and transformed fibroblasts. Nature 251: 417-419.

SCAVULLI, J., DUTTON, R.W. 1975. Competition between Con A-induced stimulatory and inhibitory effects in the in vitro immune response to antigen. J. Exp. Med. 141: 524-529.

SCHEID, M.P., HOFFMANN, M.K., KOMURO, K., HAMMERLING, U., ABBOT, J., BOYSE, E.A., COHEN, G.H., HOPPER, J.A., SCHULOF, R.S., GOLDSTEIN, A.L. 1973. Differentiation of T cells induced by preparations from thymus and by nonthymic agents. J. Exp. Med. 138: 1027-1032.

SCHIMPL, A., WECKER, E. 1972. Replacement of T cell function by a T cell product. Nature New Biol. 237: 15-17.

SCHIMPL, A., WECKER, E. 1975. A third signal in B cell activation given by TRF. Transplant. Rev. 23: 176-188.

SHEARER, G.M., MELMON, K.L., WEINSTEIN, Y., SELA, M. 1972. Regulation of antibody response by cells expressing histamine receptors. J. Exp. Med. 136: 1302-1307.

SHEPPARD, J.R. 1972. Difference in the cyclic adenosine 3':5'-monophosphate levels in normal and transformed cells. Nature New Biol. 236: 14-16.

SHEPPARD, J.R., PRASAD, K.N. 1973. Cyclic AMP levels and the morphological differentiation of mouse neuroblastoma cells. Life Sciences 12: Part II, 431-439.

SHEPPARD, H., WIGGAN, G., TSIEN, W.H. 1972. Structure-activity relationships for inhibitors of phosphodiesterase from erythrocytes and other tissues. In Advances in Cyclic Nucleotide Research, Vol. 1 (Greengard, P., Pasletti, R., Robison, G. A., eds.), p. 103-112.

SMITH, J.W., STEINER, A.L., NEWBERRY, Jr., W.M., PARKER, C.W. 1971a. Cyclic adenosine 3',5'-monophosphate in human lymphocytes. Alterations after phytohemagglutinin stimulation. J. Clin. Invest. 50: 432-441.

SMITH, J.W., STEINER, A.L., PARKER, C.W. 1971b. Human lymphocyte metabolism. Effects of cyclic and noncyclic nucleotides on stimulation by phytohemagglutinin. J. Clin. Invest. 50: 442-448.

SMITHIES, O. 1967. Antibody variability. Science 157: 267-273.

STEWARD, J.P. 1971. Immunologic responses of neonatally thymectomized rats to sheep red blood cells and to flagellin from Salmonella adelaide. Proc. Soc. Exp. Biol. Med. 138: 702-708.

STOBO, J.D., ROSENTHAL, A.S., PAUL, W.E. 1972. Functional heterogeneity of murine lymphoid lymphoid cells. I. Responsiveness to and surface binding of concanavalin A and phytohemagglutinin. J. Immunol. 108: 1-17.

STROM, T.B., BEAR, R.A., CARPENTER, C.B. 1975. Insulin-induced augmentation of lymphocyte-mediated cytotoxicity. Science 187: 1206-1208.

STROM, T.B., DEISSEROTH, A., MORGANROTH, J., CARPENTER, C.B., MERRILL, J.P. 1972. Alteration of the cytotoxic action of sensitized lymphocytes by cholinergic agents and activators of adenylate cyclase. Proc. Nat. Acad. Sci. 69: 2995-2999.

SUTHERLAND, E.W., RALL, T.W. 1957. The properties of an adenine ribonucleotide produced with cellular particles, ATP, Mg^{++}, and epinephrine or glucagon. J. Amer. Chem. Soc. 79: 3608.

SZENBERG, A., CUNNINGHAM, A.J. 1968. DNA synthesis in the development of antibody-forming cells during the early stages of the immune response. Nature 217: 747-748.

SZILARD, L. 1960. The molecular basis of antibody formation. Proc. Nat. Acad. Sci. 46: 293-302.

TALMAGE, D.W., RADOVICH, J., HEMMINGSEN, H. 1970. Cell interactions in antibody synthesis. Adv. Immunol. 12: 271-282.

TANNENBERG, W.J.K., MALAVIYA, A.N. 1968. The life cycle of antibody-forming cells. I. The generation time of 19S hemolytic plaque-forming cells during the primary and secondary responses. J. Exp. Med. 128: 895-925.

TAYLOR, R.B. 1969. Cellular cooperation in the antibody response of mice to two serum albumins: specific function of thymus cells. Transplant. Rev. 1: 114-149.

TONEGAWA, S., STEINBERG, C. DUBE, S., BERNARDINI, A. 1974. Evidence for somatic generation of antibody diversity. Proc. Nat. Acad. Sci. 71: 4027-4031.

TYAN, M.L., HERZENBERG, L.A. 1968. Studies on the ontogeny of the mouse immune system. II. Immunoglobulin producing cells. J. Immunol. 101: 446-450.

UZUNOVA, A.D., HANNA, E.E. 1973. B-cell amplification by dibutyryl cyclic AMP in mice. Cell. Immunol. 7: 507-511.

WAGNER, H. 1973. Synergy during in vitro cytotoxic allograft responses. I. Evidence for cell interaction between thymocytes and peripheral T cells. J. Exp. Med. 138: 1379-1397.

WARNER, N.L., SZENBERG, A. 1964. In The Thymus in Immunology (Good, R.A., Gabrielson, A.E., eds.) p. 395-413.

WATSON, J. 1974. The nature of the signals required for the induction of antibody synthesis. In The Immune System (Sercarz, E.E., Williamson, A.R., Fox, C.F. eds.), p.511-532.

WATSON, J. 1975. The influence of intracellular levels of cyclic nucleotides on cell proliferation and the induction of antibody synthesis. J. Exp. Med. 141: 97-111.

WATSON, J., EPSTEIN, R., COHN, M. 1973. Cyclic nucleotides as intracellular mediators of the expression of antigen-sensitive cells. Nature 246: 405-409.

WEBB, D.R., STITES, D.P., PERLMAN, J.D., LUONG, D., FUDENBERG, H.H. 1973. Lymphocyte activation: the dualistic effect of cAMP. Biochem. Biophys. Res. Commun. 53: 1002-1008.

WEINSTEIN, Y., CHAMBERS, D.A., BOURNE, H.R., MELMON, K.L. 1974. Cyclic GMP stimulates lymphocyte nucleic acid synthesis. Nature 251: 352-353.

WINCHURCH, R., ISHIZUKA, M., WEBB, D., BRAUN, W. 1971. Adenyl cyclase activity of spleen cells exposed to immunoenhancing synthetic oligo- and polynucleotides. J. Immunol. 105: 1399-1400.

WOLBERG, G., ZIMMERMAN, T.P., HIEMSTRA, K., WINSTON, M., CHU, L.C. 1975. Adenosine inhibition of lymphocyte-mediated cytolysis. Possible role of cyclic adenosine monophosphate. Science 187: 957-959.

WU, A.M., TILL, J.E., SIMINOVITCH, L., McCULLOCH, E.A. 1967. A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. J. Cell. Physiol. 69: 177-184.

YAMAMOTO, I., WEBB, D.R. 1975. Antigen-stimulated changes in cyclic nucleotide levels in the mouse. Proc. Nat. Acad. Sci. 72: 2320-2324.

APPENDIX

The Nucleotide Sequences and Coding Properties of the Major and Minor Lysine Transfer Ribonucleic Acids from the Haploid Yeast *Saccharomyces cerevisiae* αS288C